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<p>(21) International Application Number: PCT/AU89/00123</p> <p>(22) International Filing Date: 23 March 1989 (23.03.89)</p> <p>(31) Priority Application Number: PI 7391</p> <p>(32) Priority Date: 23 March 1988 (23.03.88)</p> <p>(33) Priority Country: AU</p> <p>(71) Applicant (for all designated States except US): THE UNIVERSITY OF MELBOURNE [AU/AU]; Parkville, VIC 3052 (AU).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only) : SINGH, Mohan, Bir [IN/AU]; 7 Lloyd Court, Templestowe, VIC 3106 (AU). HOUGH, Terryn [AU/AU]; 25 Bowman Street, Mordialloc, VIC 3195 (AU). THEERAKULPISUT, Piyada [TH/AU]; 1/74 Canning Street, Carlton, VIC 3053 (AU). KNOX, Robert, Bruce [AU/AU]; 274 Balwyn Road, North Balwyn, VIC 3104 (AU).</p>		<p>(74) Agents: NOONAN, Gregory, J. et al.; Davies & Collison, 1 Little Collins Street, Melbourne, VIC 3000 (AU).</p> <p>(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), DK, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.</p> <p>Published With international search report.</p>
<p>(54) Title: RYEGRASS POLLEN ALLERGEN</p> <p>(57) Abstract</p> <p>The major allergenic protein <i>LOI pI</i> from pollen of ryegrass <i>Lolium perenne</i>L. is produced by recombinant DNA techniques. The DNA sequence encoding the above protein, expression vectors, host-transformed and cell lines containing the coding sequence for <i>LOI pI</i> protein are also described. The use of the above DNA sequences and recombinant protein in nucleic hybridization, tissue specificity diagnosis and detection of specific antibodies in biological samples are also disclosed. The possible use of the promoter sequence of <i>LOI pI</i> in the developmental regulation of <i>LOI pI</i> gene expression or any other gene during the development of the pollen, in inhibiting pollen development or function and inducing nuclear male sterility are also disclosed.</p>		

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1 "RYEGRASS POLLEN ALLERGEN"

3 The present invention relates to the major
allergenic protein Lol pI from pollen of ryegrass,
5 Lolium perenne L. and to derivatives and homologues
thereof and to allergenic proteins immunologically related
7 thereto. More particularly, the present invention is
directed to recombinant Lol pI, and its derivatives, and
9 to an expression vector capable of directing synthesis of
same. Even more particularly, the present invention is
11 directed to cDNA encoding Lol pI and to its promoter and
to an expression vector comprising same.

13

Allergens constitute the most abundant proteins of
15 grass pollen, which is the major cause of allergic disease
in temperate climates (MARSH, 1975, HILL et al., 1979).
17 The first descriptions of their allergenic proteins showed
that they are immunochemically distinct, and are known as
19 groups I, II, III and IV (JOHNSON AND MARSH 1965, 1966).
Using the recently proposed International Union of
21 Immunological Societies' (IUIS) nomenclature, these
allergens are designated Lol pI, Lol pII, Lol pIII and
23 Lol pIV. The major allergen, Lol pI is an acidic
glycoprotein of molecular weight ca.32 kD and comprises
25 four isoallergenic variants. The other minor allergens
isolated from ryegrass pollen range in molecular weight
27 from 10 to 76 kD (see review by FORD AND BALDO, 1986). The
allergen Lol pI constitutes ca.5% of the total extracted
29 pollen proteins and is a glycoprotein (HOWLETT & CLARKE,
1981) containing a 5% carbohydrate moiety. Studies with
31 carbohydrate splitting have demonstrated that the
carbohydrate does not contribute to the allergic
33 response. Allergenic activity is lost following

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1 proteolytic digestion (see review by BALDO, SUTTON &
2 WRIGLEY, 1982), but is resistant to heat treatment, e.g.
3 100°C for 30 minutes at neutral pH (MARSH et al., 1966).

5 Lol pI is defined as an allergen because of its
6 ability to bind to specific IgE in sera of
7 ryegrass-sensitive patients, to act as an antigen in IgG
8 responses, and to trigger T-cell responses. The
9 allergenic properties have been assessed by direct skin
10 testing of grass pollen-sensitive patients. The results
11 showed that 84% had a skin sensitivity to Lol pI
12 (FREIDHOFF et al., 1986), demonstrating the primary
13 importance of this protein as the major allergen.
14 Furthermore, 95% of patients demonstrated to be grass
15 pollen-sensitive possessed specific IgE antibody that
16 bound to Lol pI, as demonstrated by immunoblotting (FORD &
17 BALDO, 1986).

19 Substantial allergenic cross-reactivity between
20 grass pollens has been demonstrated using an IgE-binding
21 assay, the radioallergo-sorbent test (RAST), for example,
22 as described by MARSH (1970) and LOWENSTEIN (1978).

23

24 The immunochemical relationships of Lol pI with
25 other grass pollen antigens have been demonstrated using
26 both polyclonal and monoclonal antibodies (e.g. SMART &
27 KNOX, 1979; SINGH & KNOX, 1985). Antibodies have been
28 prepared to both purified proteins and IgE-binding
29 components. These data demonstrate that the major
30 allergen present in pollen of closely related grasses is
31 immunochemically similar to Lol pI (SINGH & KNOX, 1985).

33 Further background information concerning grass
34 pollen allergens can be found in the following reviews:

- 3 -

1 MARSH (1975), HOWLETT & KNOX (1984), BALDO, SUTTON &
WRIGLEY (1982) and FORD & BALDO (1986).

3 Recent advances in biochemistry and in recombinant
DNA technology have made it possible to synthesize
5 specific proteins, for example, enzymes, under controlled
conditions independent of the organism from which they are
7 normally isolated. These biochemical synthetic methods
employ enzymes and subcellular components of the protein
9 synthesizing systems of living cells, either in vitro in
cell-free systems, or in vivo in microorganisms. In
11 either case, the principal element is the provision of a
deoxyribonucleic acid (DNA) of specific sequence which
13 contains the information required to specify the desired
amino acid sequence. Such a specific DNA sequence is
15 termed a gene. The coding relationships whereby a
deoxyribonucleotide sequence is used to specify the amino
17 acid sequence of a protein is well-known and operates
according to a fundamental set of principles (see for
19 example, WATSON, 1976).

21 A cloned gene may be used to specify the amino acid
sequence of proteins synthesized by in vitro systems.
23 DNA-directed protein synthesizing systems are
well-established in the art. Single-stranded DNA can be
25 induced to act as messenger RNA (mRNA) in vitro, thereby
resulting in high fidelity translation of the DNA sequence.

27

It is now possible to isolate specific genes or
29 portions thereof from higher organisms, such as plants,
and to transfer the genes or DNA fragments in a suitable
31 vector, such as lambda-gt 11 phage, to microorganisms such
as bacteria e.g. Escherichia coli. The transferred gene
33 is replicated and propagated as the transformed
microorganism replicates. Consequently, the transformed

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1 microorganism is endowed with the capacity to make the
desired protein or gene which it encodes, for example, and
3 enzyme, and then passes on this capability to its
progeny. See, for example, Cohen and Boyer, U.S. Patent
5 Nos. 4,237,224 and 4,468,464. The bacterial clones
containing the recombinant phage are screened for the
7 particular gene product (protein) by means of specific
antibodies.

9

In accordance with the present invention, the gene
11 encoding Lol pI is cloned and thereby permitting the large
scale production of recombinant allergen.

13

Accordingly, one aspect of the present invention
15 relates to a recombinant vector comprising a DNA sequence
encoding a protein displaying allergenic activity from
17 pollen of a grass species. More particularly, the grass
species belongs to the family Poaceae (Gramineae), and
19 even more particularly, to the genus Lolium. Still even
more particularly, the allergenic protein is characterized
21 as being immunologically cross-reactive with antibody to
Lol pI protein of Lolium perenne pollen, namely:

23

Pooid (festucoid) grasses. GROUP 1: Triticanae:
25 Bromus inermis, smooth brome; Agropyron repens, English
couch; A.cristatum; Secale cereale rye Triticum
27 aestivum, wheat. GROUP 2: Poanae: Dactylis glomerata,
orchard grass or cocksgoot; Festuca elatior, meadow
29 fescue; Lolium perenne, perennial ryegrass;
L.multiflorum, Italian ryegrass; Poa pratensis, Kentucky
31 bluegrass; P.compressa, flattened meadow grass; Avena
sativa, oat; Holcus lanatus, velvet grass or Yorkshire
33 fog; Anthoxanthum odoratum, sweet vernal grass;
Arrhenatherum elatius, oat grass; Agrostis alba, red

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1 top; Phleum pratense, timothy; Phalaris arundinacea,
reed canary grass. Panicoid grass, Paspalum notatum,
3 Bahia grass, Andropogonoid grasses: Sorghum halepensis,
Johnson grass.

5

Another aspect of the present invention relates to a
7 recombinant vector comprising a DNA sequence encoding the
allergenic protein Lol pI of ryegrass, Lolium perenne, L.
9 pollen, or a derivative or homologue thereof. More
particularly, the present invention relates to a
11 recombinant DNA molecule comprising a eukaryotic or
prokaryotic origin of replication, a detectable marker, a
13 DNA sequence encoding the Lol pI allergenic protein or a
derivative or a homologue thereof or an allergenic protein
15 cross-reactive with said Lol pI protein or its derivatives
or homologues and optionally a promoter sequence capable
17 of directing transcription of said allergenic protein.

19 Yet another aspect of the present invention
contemplates a method for producing recombinant Lol pI or
21 a derivative or homologue thereof or an allergenic protein
immunologically reactive to antibodies to Lol pI or a
23 derivative or homologue thereof, comprising culturing an
organism containing a replicable recombinant DNA molecule,
25 said molecule comprising a promoter capable of expression
in said organism, the gene encoding Lol pI or its
27 derivative or homologue or immunologically related protein
of Lol pI located downstream of and transcribed from said
29 promoter, a selectable marker and a DNA vehicle containing
a prokaryotic or eukaryotic origin of replication, under
31 conditions and for a time sufficient for said recombinant
DNA molecule to be stably maintained and direct the
33 synthesis of Lol pI or its derivative or homologue.

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1 In yet another aspect of the present invention,
there is provided non-native (i.e., recombinant or
3 chemically synthesized) Lol pI or its derivative or
homologue or a non-native allergenic protein
5 immunologically cross-reactive to antibodies to Lol pI or
its derivative or homologue .

7

Still yet another aspect of the present invention
9 relates to antibodies to non-native Lol pI or a derivative
or homologue thereof.

11

In still yet another aspect of the present
13 invention, there is provided a method for detecting an
antibody to an allergenic protein from pollen of the
15 family Poaceae (Gramineae) in serum or other biological
fluid comprising contacting said serum or fluid with
17 recombinant Lol pI or its antigenic derivative for a time
and under conditions sufficient for an antibody - Lol pI
19 complex to form and subjecting said complex to a detecting
means.

21

Another aspect of the present invention relates to a
23 recombinant DNA molecule comprising a ryegrass pollen
promoter sequence or homologue or degenerate form thereof
25 located on said molecule and further having one or more
restriction sites down stream of said promoter such that a
27 nucleotide sequence inserted into one or more of these
sites is transcribeable in the correct reading frame.

29

In one preferred embodiment, the recombinant DNA
31 molecule comprises the promoter directing synthesis of
Lol pI from pollen of ryegrass, Lolium perenne L. and is
33 thereby a developmentally regulated, pollen specific,
expression vector.

35

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1 A further aspect of the present invention
contemplates a method for inducing nuclear male sterility
3 in plants of the family Poaceae comprising the steps of:

a) developing a plant carrying a recombinant DNA
5 molecule comprising the ryegrass pollen promoter sequence
or homologue or degenerate form thereof located on said
7 molecule and a nucleotide sequence encoding a polypeptide
having a deleterious function in cells derived from the
9 family Poaceae, said nucleotide sequence transcribeable
from said promoter, and said recombinant DNA molecule
11 stably contained in pollen producing cells, and,

b) growing said plants under conditions and for a
13 time sufficient for their developmental stage to cause
expression of said nucleotide sequence from said promoter
15 thereby producing the polypeptide having a deleterious
function on said pollen producing cells such that pollen
17 formation is inhibited or said pollen is inactive.

19 Further features of the present invention will be
better understood from the following detailed description
21 of the preferred embodiments of the invention in
conjunction with the appended figures.

23

Standard biochemical nomenclature is used herein in
25 which the nucleotide bases are designated as adenine (A);
thymine (T); guanine (G) and cytosine (C). Other
27 abbreviations include:-

29 BSA	Bovine serum albumin.
DEPC	Diethyl pyrocabonate.
31 DNA	Deoxyribonucleic acid.
DTT	Dithiothreitol.
33 EDTA	Disodium ethylene diamine tetra-acetate.
IP TG	Isopropyl-thio-beta-D-galactopyranoside.

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- 1
LB MEDIUM Luria-Bertani medium (1% (w/v) Bactotryptone,
3 1% (w/v) NaCl & 0.5% (w/v) Bacto-yeast
 extract in water to pH 7.5).
- 5
LIGATION BUFFER (10 x solution)
7 0.66 M Tris Cl (pH 7.5) 50mM Mg Cl₂, 50mM
 DTT, 10mM ATP.
- 9
LiCl Lithium chloride.
- 11 PEG Polyethylene glycol.
 pfu Plaque forming units.
- 13 PMSF Phenylmethylsulphonylfluoride.
- 15 SAMPLE BUFFER
 50mM Tris-Cl, pH 6.8, 1.5% (w/v) SDS, 50mM
17 DTT, 4M Urea, 1m MPMSF.
- 19 SDS Sodium dodecyl sulfate.
- 21 SM BUFFER Phage storage buffer (0.1M NaCl, MgSO₄7H₂O,
 50mM Tris HCl pH 7.5, 2% (w/v) gelatin).
- 23
SSC 20 x solution of 3M NaCl, 0.3M Na₃ citrate,
25 pH 7.0.
- SSPE (0.15M NaCl, 10mM Sodium Phosphate pH 7.7, 1mM
- 27 EDTA Ethylene diamine tetra-acetic acid
- TBS Tris buffered saline (50mM Tris pH 7.5, 150
29 mM NaCl).
- X-gal 5-Bromo-4-chloro 3-indolyl
31 beta-D-galactopyranoside.

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1 In the accompanying figures:

3 Figure 1 shows the identification of Lol pI as the
principal allergen of ryegrass pollen by SDS-PAGE and
5 Western Blotting.

7 Lanes 1-3. SDS-PAGE analysis of total ryegrass
pollen proteins and isolated Lol pI allergen, stained with
9 Coomassie blue for proteins. Lane 1, total pollen
proteins; Lane 2, isoallergen of Lol pI; Lane 3, Lol pI.
11

Lanes 4-6. Western blot of proteins shown in Lanes
13 1-3, showing specific binding of monoclonal antibody
FMC-A1 to the Lol pI allergen. This antibody was used to
15 screen the cDNA library to select the Lol pI allergen
clones. Lane 4, molecular markers; Lane 5, isoallergen;
17 Lane 6, Lol pI, 32 kD.

Figure 2 shows screening of cDNA library of ryegrass
19 pollen to select the specific clones which express Lol pI
protein in lambda-gt 11 vector. (a,b): Plaque - lifts of
21 $10^2 - 10^3$ recombinant phages treated with specific
antibody FMC-A1, with (a) cDNA clone 6; (b) cDNA clone
23 12; (c,d) Re-screening of cDNA clone 12 with (c)
monoclonal antibody FMC-a1, (d) specific IgE from ryegrass
25 pollen-sensitive patients' sera. Recombinant phage
containing the specific allergen DNA insert are detected
27 by these methods. The antibodies detect all clones which
contain the antigenic determinants of Lol pI, while IgE
29 binds to clones containing the allergenic determinants of
Lol pI. All clones were monoclonal antibody FMC-A1
31 positive, as this is the basis of the screen, while a
proportion bind to IgE, as with clone 12 here.

33

Figure 3 shows analysis of E. coli fusion protein
35 for identity with Lol pI.

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1

Lanes 1-4: SDS-PAGE stained with Coomassie blue for
3 proteins; Lane 1, lambda-gt 11 non-recombinant lysogen
extract; Lane 2, lambda-l2RL8 recombinant lysogen
5 extract; Lane 3, *E. coli* beta-galactosidase pure
protein; Lane 4, molecular weight markers, the 96KD
7 marker is indicated by a star.

Lanes 5-7: Transblots on nitrocellulose membrane;
9 Lane 5 and 8, molecular weight markers; Lane 6,
lambda-l2RL8 recombinant lysogen extract, a indicates a
11 fusion protein identified by binding with FMC-A1,
molecular weight a>c. Lane 7, lambda-6RL2 recombinant
13 lysogen extract, B indicates fusion protein as identified
by binding of FMC-A1, molecular weight b>c. The higher
15 molecular weights of a and b over c indicates the
insertion of cDNA into the gt 11 genome at the lacZ site.

17

Figure 4 shows analysis of tissue and organ
19 specificity of *Lol* pI gene in ryegrass. (a) Slot blot.
2ug each of total RNA isolated from pollen (p), leaf (l),
21 roots (r) and hydrated seeds (s) were slot blotted onto
nitrocellulose membrane. Hybridization with radioactive
23 probes for clones 6 and 12 (p6, p12) occurs with pollen,
but there is a total absence of hybridization with the
25 other tissue RNA. (b) Northern blot. Total RNA isolated
from these ryegrass tissues were separated
27 electrophoretically in a denaturing agarose gel, and
transferred to nitrocellulose membrane, and probed with
29 p6. Hybridization occurs with the pollen sample only,
other tissue RNA showing absence of hybridization. This
31 evidence shows that *Lol* pI gene is expressed only in
pollen.

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1

Figure 5 shows a 1240 base pair DNA sequence
3 representing the cDNA clone 12R

5 Figure 6 shows the reaction of recombinant allergen
pGEX-12R (Lol pI) with IgE from pooled allergic sera. The
7 cultures of pGEX and pGEX-12R were grown overnight and
then diluted 1:10 in broth and grown for 2h at 37°C. They
9 were induced with IPTG, and grown for 1h at 37°C. The
bacteria were pelleted and resuspended in PBS to 1/20 the
11 volume of culture media. The bacteria were lysed by
freeze thaw and sonication. Following that an equal
13 volume of SDS gel sample buffer was added, and samples
boiled for 3 min, before loading them onto a 10-15%
15 gradient SDS-PAGE. The separated proteins were
transferred onto nitrocellulose membrane, and these blots
17 were processed for identification of IgE-binding proteins
using pooled sera from allergic patients. ¹²⁵I-labelled
19 anti-human IgE antibodies (Kallestad Labs USA) were used
as probe. Figure 6 shows a typical autoradiograph in
21 which lane 1 shows a vector control in which no IgE
binding is present, while lanes 2,3 and 4 show expression
23 of recombinant Lol pI in bacterial cultures infected with
pGEX-12R.

25

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1 Figure 7 A,B and C shows antigenic and allergenic
2 similarity of proteins homologous with Lol pI in a panel
3 of 17 different grasses. Proteins were resolved by
4 SDS-PAGE from mature pollen as follows: lane a: molecular
5 weight markers; 1, Bromus inermis; 2, Agropyron
6 cristatum; 3, Secale cereale; 4, Dactylis glomerata; 5,
7 Festuca elatior; 6, Lolium perenne; 7, L. multiflorum;
8, Poa compressa; 9, Avena sativa; 10, Holcus lanatus;
9 11, Anthoxanthum odoratum; 12, Agrostis alba; 13, Phleum
10 pratense; 14, Phalaris arundinacea; 15, Cynodon
11 dactylon; 16, Sorghum halepensis; 17, Zea mays. 7A shows
12 Coomassie blue stained proteins in SDS-PAGE gel. 7B shows
13 western blot probed with monoclonal antibody FMC-A1
14 specific for Lol pI, showing antigenic similarity of Lol
15 pI and homologous allergens in related grasses, except for
16 lane 15, Cynodon dactylon. 7C shows western blot probed
17 with pooled allergic human sera and anti-IgE antibodies,
18 confirming that Lol pI and its homologous allergens in
19 other grasses are the immunodominant allergen of grass
20 pollen.

21

22 Figure 8A shows a comparison of allergenic activity
23 of native and recombinant Lol pI protein. Sera from 28
24 different patients, some of whom are allergic to grass
25 pollen, were used to compare the IgE binding of native and
26 recombinant Lol pI protein. For native Lol pI, a
27 reference standard sample was purchased from the National
28 Institutes of Health (NIAID), Bethesda, USA. This sample
29 was diluted in 1% (w/v) BSA solution, and 0.5ug was
30 dot-blotted onto nitrocellulose membrane, and the blots
31 used for IgE-binding assay. For testing IgE-binding to
32 recombinant Lol pI protein, the clone lambda-gt 11 -12R
33 was expressed in host E. coli cells. The plaque lifts
were used in a similar way to dot blots for testing IgE

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1 binding. Both the plaque lifts and dot blots were
incubated overnight in 1:10 dilution of allergic sera, and
3 binding of IgE visualized using rabbit anti-human IgE
(Dakopatts, Copenhagen, Denmark). This incubation was
5 followed by peroxidase-conjugated goat anti-rabbit IgG,
and then the enzyme substrate to give a colour reaction.
7 Figure 8B is a correlation of allergenic reactivity of
native and recombinant Lol pI.

9

Figure 9 shows restriction map of cDNA insert to
11 lambda-gt 11 -12R, and the strategy of nucleotide
sequencing.

13

In accordance with the present invention, there is
15 provided the gene encoding the ryegrass pollen allergen
Lol pI, a method for expressing same in a host cell, and
17 more particularly organ specific (i.e., pollen), thereby
providing a source of recombinant Lol pI and the promoter
19 of the Lol pI gene directing developmental regulation of
Lol pI or any genetic sequence placed downstream thereof.

21

The original source of the genetic material is fresh
23 ryegrass pollen from Lolium perenne L., collected from
field sources near Melbourne, Australia and bulk collected
25 pollen from a supplier (Greer Laboratories, Lenoir, NC).
These sources of pollen are not intended to limit the
27 scope of the invention since they only represent one
convenient supply of the pollen. The present invention
29 can be practised using pollen from any location. Figure 1
shows the identification of Lol pI as the principle
31 allergen of ryegrass pollen.

33 "Gene", is used, in respect of the present
invention, in its broadest sense and refers to any
35 contiguous sequence of nucleotides, the transcription of
which, leads to a mRNA molecule, whether or not said mRNA

- 14 -

1 molecule is translatable into a polypeptide or protein.
The gene encoding Lol pI means the nucleotide sequence
3 encoding the entire polypeptide or derivatives or
homologues of said polypeptide which may contain amino
5 acid substitutions, deletions or additions. Similarly, in
relation to the carbohydrate portion of said polypeptide,
7 derivatives include substitutions, deletions or additions
to said carbohydrate moiety. The Lol pI gene also refers
9 to a cDNA complementary to the mRNA corresponding to the
full or partial length of the Lol pI polypeptide.
11 Accordingly, it is within the scope of the present
invention to encompass Lol pI and its amino acid and/or
13 carbohydrate derivatives and to nucleotide sequences,
including DNA, cDNA and mRNA and to the homologue or
15 degenerate forms thereof, encoding said Lol pI or said
derivatives. It is further in accordance with the present
17 invention to include molecules such as polypeptides fused
to Lol pI or its derivatives or to nucleotide sequences
19 contiguous to the Lol pI- and/or derivative-encoding
nucleotide sequences. For example, for some aspects of
21 the present invention, it is desirable to produce a fusion
protein comprising Lol pI or its derivative and an amino
23 acid sequence from another polypeptide or protein,
examples of the latter being enzymes such as
25 beta-galactosidase, phosphatase, urease and the like.
Most fusion proteins are formed by the expression of a
27 recombinant gene in which two coding sequences have been
joined together such that their reading frames are in
29 phase. Alternatively, polypeptides can be linked in vitro
by chemical means. All such fusion protein or hybrid
31 genetic derivatives of Lol pI or its encoding nucleotide
sequence are encompassed by the present invention.
33 Furthermore, by homologues and derivatives of Lol pI is
meant to include synthetic

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1 derivatives thereof. The nucleotide sequence as
2 elucidated herein, can be used to generate any number of
3 peptides or polypeptides by chemical synthesis, such as
4 solid phase synthesis, by well known methods. All such
5 chemically synthesized peptides are encompassed by the
6 present invention. Accordingly, the present invention
7 extends to non-native Lol pI, and its derivatives,
8 homologues and immunological relatives made by recombinant
9 means or by chemical synthesis. Furthermore, the present
10 invention extends to proteins, polypeptides or peptides
11 corresponding in whole or part to the nucleotide coding
12 sequence given in Figure 5 or to degenerate or homologue
13 forms thereof.

14 It is also within the scope of the present invention
15 to include allergenic proteins immunologically
16 cross-reactive with antibodies to Lol pI or its
17 derivatives or homologues. "Immunologically
18 cross-reactive" is used in its broadest sense and refers
19 generally to a protein capable of detectable binding to an
20 antibody, the latter being specific to Lol pI or to
21 derivatives or homologues of Lol pI. Such an
22 immunologically related allergen is referred to herein as
23 a immunological relative of Lol pI.

25

26 The cloning of the cDNA encoding Lol pI was based on
27 the recognition of the protein expressed by Escherichia
28 coli transformed with lambda-gt 11 phage, using both
29 specific monoclonal antibodies and specific serum IgE from
30 grass pollen-sensitive patients. Two such clones are
31 designated 6R and 12R. cDNA clones were also isolated on
32 the basis of differential antibody binding. For example,
33 cDNA clone 6R, was isolated on the basis that it encoded a
34 polypeptide capable of binding to monoclonal antibodies
35 but not IgE. Polypeptides of this type apparently lack
36 the amino acid sequence specifying allergenicity and
37 hence, these cDNA clones must lack the DNA sequence
38 encoding same. Monoclonal antibodies used herein are FMC
39 A1, A5 and A7 as described by KNOX & SINGH (1985).

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1

For cloning the Lol pI gene or derivatives thereof,
3 mRNA was first isolated from the mature ryegrass pollen.
This mRNA was used as a template to synthesize double
5 stranded complementary DNAs

7

From this cDNA library of ryegrass pollen
recombinant phage containing the Lol pI insert were
9 detected by screening the library with (1) specific
monoclonal antibody FMC-A1; (2) specific IgE from sera of
11 ryegrass-sensitive patients (Fig. 2).

13

EcoRI, linkers were then attached to both sides of
selected clones of ds cDNA and then ligated into EcoRI,
15 lambda-gt 11 vector arms (cut and dephosphorylated as
purchased from Promega). The recombinant lambda-gt 11 DNA
17 containing cDNA inserts were packaged into mature phage
and the recombinant phage allowed to infect the E. coli
19 host.

21

The synthesis of beta-galactosidase - recombinant
gene fusion protein was induced by adding IPTG. The
23 Lol pI-beta-galactosidase fusion protein was then detected
using monoclonal antibodies which specifically recognise
25 the epitopes on Lol pI protein.

27

This fusion protein was isolated in preparative
amounts from bacterial lysogens, fractionated by
29 SDS-polyacrylamide gel electrophoresis, and the proteins
transferred to nitrocellulose membranes for probing with
31 monoclonal antibodies (Fig. 3). These antibodies
recognised a protein which shows a molecular weight
33 greater than the E. coli beta-galactosidase as would be
expected of an allergen beta-galactosidase fusion protein.

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1

The allergenic nature of the subject proteins are characterised in part, by their binding of the reagenic IgE antibodies which are present at high levels in sera of allergic patients. The IgE binding to the epitopes on allergic proteins can be tested in a chromogenic assay in which allergens immobilized on a solid support can be visualised by sequential incubation in (1) allergic patients serum; (2) enzyme-labelled anti-IgE antibodies.

Selected cDNA clones were used to probe total RNA isolated from other ryegrass plant organs to test whether Lol pI allergen is pollen-specific or not. Slot-blotting and Northern analyses were employed (Fig. 4). No hybridization was detectable for total RNA from leaf, seed or root samples. These data indicate that Lol pI is not expressed in these other organs of the ryegrass plant.

Selected cDNA clones were ligated into both M13 and Gemini vectors for sequencing. DNA restriction fragments to be sequenced were inserted into M13 mp14 (MESSING AND VIEIRA 1982). M13 cloning and dideoxy chain termination DNA sequencing were performed as described by Bio-rad Laboratories (1980) and MESSING (1983). A similar approach is used for the cloning of allergenic proteins from pollen of other members of the family Poaceae (Gramineae) which are immunologically cross-reactive with antibodies to Lol pI or its derivatives or homologues. The sequence of the 1240 base pair cDNA clone 12R is shown in Figure 5. It is in accordance with this invention to include or degenerate forms of said sequence and/or nucleotide sequences having substantial i.e., at least 60% homology thereto.

With this knowledge in hand, a variety of expression vectors can be constructed for the production of Lol pI or its derivatives. Accordingly, another aspect of the present invention contemplates a method of producing recombinant Lol pI or its derivative or homologue or its

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1 immunological relative (as hereinbefore defined)
2 comprising culturing an organism containing a replicable
3 recombinant DNA molecule, said molecule comprising a
4 promoter capable of expression in said organism, the
5 Lol pI gene or gene encoding its derivative, homologue or
6 immunological relative thereof, located downstream of and
7 transcribed from said promoter, a selectable marker and a
8 DNA vehicle containing a prokaryotic or eukaryotic origin
9 of replication, under conditions and for a time sufficient
10 for said recombinant DNA molecule to be stably maintained
11 and direct the synthesis of Lol pI or its derivative,
12 homologue or immunological relative and then isolating
13 same.

15 "Promoter" is used in its broadest sense and refers
16 generally to nucleotide sequence which binds RNA
17 polymerase and directs same to the correct transcriptional
18 start site whereupon a gene or other nucleotide sequence
19 thereof is transcribed. As used herein, a gene or
20 nucleotide sequence is said to be relative to the promoter
21 meaning that said promoter directs the transcription of
22 the gene or nucleotide sequence. The promoter is also
23 selected on the basis of its ability to function in a
24 particular host. The following description relates to
25 developing prokaryotic expression vectors capable of
26 expressing the Lol pI gene or a gene encoding its
27 derivative, homologue or immunological relative, thereof.
28 Similar principles apply for the construction of
29 eukaryotic vectors. In this description, reference to the
30 Lol pI gene also includes reference to genes encoding
31 derivative, homologues or immunological relatives of
Lol pI.

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1

In constructing suitable prokaryotic expression vectors, transcription termination sequences are desirable to prevent potential readthrough by the RNA polymerase. To avoid any potential interference with the transcription terminators, one skilled in the art can eliminate the 3' non-coding region of the Lol pI gene. Concurrently, one can substitute other known transcription terminators, for example, the bacteriophage lambda terminator. Thus, the present invention is in no way limited to the use of any one prokaryotic transcription terminator. Other transcription terminators include, for example, the lpp terminator and the phage SP01 terminator. All of the aforementioned terminators have been previously characterized, are well known in the art, and can be constructed either synthetically or from known plasmids.

17

Expression of Lol pI activity in E. coli is in no way limited to the use of a particular promoter, since the choice of a specific promoter is not critical to the operability of this aspect of the present invention.

Promoters which can be substituted for the previously exemplified λP_L promoter include, but are not limited to, the E. coli lactose (lac), the E. coli tryptophan (trp), the E. coli lipoprotein (lpp), and bacteriophage lambda P promoters. In addition, one or more promoters can be used in tandem, such as, for example, the trp and lac promoters, or hybrid promoters, such as the tac promoter, can be used to drive expression of the Lol pI gene. All of the aforementioned promoters have been previously characterized, are well known in the art, and can be constructed either synthetically or from known plasmids.

33

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1 The present invention is not limited to the use of
any particular prokaryotic replicon. Many replicons, such
3 as those from plasmids pBR322, pACYC184, the pUC plasmids,
and the like, are known in the art and are suitable for
5 the construction of recombinant DNA cloning and expression
vectors designed to drive expression of the Lol pI
7 -encoding DNA compounds of the present invention. Neither
is the present invention limited to the actual selectable
9 markers present on the plasmids exemplified herein. A
wide variety of selectable markers exist, both for
11 eukaryotic and prokaryotic host cells, that are suitable
for use on a recombinant DNA cloning or expression vector
13 comprising a DNA compound (or sequence) of the present
invention.

15 Many modifications and variations of the present
17 illustrative DNA sequences and plasmids are possible. For
example, the degeneracy of the genetic code allows for the
19 substitution of nucleotides throughout polypeptide coding
regions as well as for the substitution of the TAA or TGA
21 ATT ACT
translation stop signals for the TAG translational stop
23 ATC
signal. Such sequences can be deduced from the amino acid
25 or DNA sequence of Lol pI and can be constructed by
following conventional synthetic procedures. Therefore,
27 the present invention is no way limited to the DNA
sequences and plasmids specifically exemplified.

29

The practice of this invention using prokaryotic
31 expression vectors as well as the methods disclosed in
this invention can be applied to a wide range of host
33 organisms, especially Gram-negative prokaryotic organisms
such as Escherichia coli, E. coli K12, E. coli K12 RV308,

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1 E. coli K12 HB101, E. coli K12 C600, E. coli K12 SF8,
2 E. coli K12 RR1, E. coli K12 RR1 M15, E. coli K12 MM294,
3 E. coli SG936, and the like. Escherichia coli SG936 is
4 disclosed in BUELL et al. (1985). Two of the genetic
5 mutations introduced in this strain, the lon and htpR
6 mutations are known to promote the expression of desired
7 proteins (see, for example, GOFF and GOLDBERG (1985).
8 These mutations can be transduced into other strains of
9 E. coli by P1 transduction according to the teaching of
10 MILLER (1972).

11

12 Alternatively, other prokaryotes can be readily
13 employed such as Bacillus, Pseudomonas and the like.
14 Minor modifications will need to be made to the expression
15 vector depending on the host cell employed so that the
16 vector replicates, the promoter functions and the
17 selectable marker is expressed. Such modifications would
18 be routine for one skilled in the art.

19

20 Similar considerations apply in developing
21 eukaryotic expression vectors and many are available for
22 use in mammalian cells, yeast and fungal cells and insect
23 cells. A convenient reference guide to developing
24 eukaryotic or prokaryotic expression vectors can be found
25 in MANIATIS et al. (1982)

26 The present invention also extends to the promoter
27 of ryegrass pollen proteins, and particularly, to the
28 promoter of the Lol pI gene. This promoter
29 developmentally regulates Lol pI gene expression and is
30 organ, i.e., pollen specific. Developmental regulation as
31 used herein refers to the expression of a particular
32 trait, in this case allergenic proteins in pollen, during
33 a certain stage in a plants life cycle and non-expression
34 during another stage.

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1 Hence, the Lol pI promoter is particularly useful in
allowing expression of Lol pI, or any other gene or
3 nucleotide sequence relative thereto, only during the
development of pollen. The skilled artisan will
5 immediately recognise the importance of such a promoter in
selectively expressing a particular trait during pollen
7 formation.

9 Accordingly, the present invention contemplates a
method of inhibiting pollen development or function and
11 thereby inducing nuclear male sterility in plants of the
family Poaceae, and in particular Lolium perenne L.,
13 comprising the steps of:

a) developing a plant carrying a recombinant DNA
15 molecule comprising the ryegrass pollen promoter sequence
or homologue or degenerate form thereof located on said
17 molecule and a nucleotide sequence encoding a polypeptide
having a deleterious function in cells derived from the
19 family Poaceae, said nucleotide sequence transcribeable
from said promoter, and said recombinant DNA molecule
21 stably contained in pollen producing cells, and,

b) growing said plants under conditions and for a
23 time sufficient for their developmental stage to cause
expression of said nucleotide sequence from said promoter
25 thereby producing the polypeptide having a deleterious
function on said pollen producing cells such that pollen
27 formation is inhibited or said pollen is inactive.

29 Well established methods exist for introducing
recombinant DNA molecules into plant cells such as use of
31 Agrobacterium plasmids and electroporation amongst
others. By "deleterious function" in respect of a
33 polypeptide refers to a feature of said polypeptide that
will inhibit cell growth, cause lysis of a cell, or

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1 inhibit various functions in a cell and thereby preventing
the normal functioning of the cell. In this case, lethal
3 gene constructs having a deleterious function are
contemplated which inhibit or prevent pollen formation and
5 thereby result in a male sterile plant. Such "lethal
genes" may encode enzymes, enzyme inhibitors, and/or toxic
7 polypeptides, amongst other molecules. Alternatively, the
lethal gene may encode an antisense RNA capable of
9 inhibiting translation of a particular species of mRNA,
the translated product thereof, being vital for pollen
11 development.

13 Male sterile plants are particularly useful in
developing hybrid crop varieties.

15

The Lol pI promoter is isolatable from ryegrass
17 genomic DNA by any number of procedures including use of
promoter probes vectors, "chromosome walking" and S1
19 nuclease mapping and sequencing as DNA upstream of the
transcription initiation site. All these techniques are
21 well known to the skilled artisan. For example, using the
cDNA clone encoding Lol pI or its derivative as probe DNA
23 for hybridization, a fragment of DNA adjacent to or
encompassing part or all of the Lol pI gene is cloned.
25 The nucleotide sequence of Lol pI as determined in
accordance with the present invention, is then used, to
27 develop nucleotide primers at the promoter-proximal end of
the Lol pI gene. "Chromosome walking", S1 endonuclease
29 mapping, promoter probes will readily identify the
promoter.

31

Accordingly, the present invention contemplates a
33 recombinant DNA molecule comprising a ryegrass pollen
promoter sequence, and in particular the promoter for the

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1 Lol pI gene, or homologue or degenerate form thereof
located on said molecule and further having one or more
3 restriction endonuclease sites downstream of said promoter
such that nucleotide sequence inserted into one or more of
5 these sites is transcribeable in the correct reading
frame. As used herein, the "correct reading frame" has
7 the same meaning as "in phase". The aforementioned DNA
molecule will preferably also have a selectable marker
9 thereon, such as an antibiotic or other drug resistance
gene, such as for example gene encoding resistance to
11 ampicillin, carbenicilin, tetracycline, streptomycin and
the like. The recombinant molecule will further comprise
13 a means for stable inheritance in a prokaryotic and/or
eukaryotic cell. This can be accomplished by said
15 recombinant molecule carrying a eukaryotic and/or a
prokaryotic origin of replication as hereinbefore
17 described in relation to expression vectors.

Alternatively, the recombinant molecule will carry a means
19 for integration into a host cell genome thereby permitting
replication of said recombinant molecule in synchrony with
21 the replication of said host cell genome. Examples of
preferred prokaryotic hosts include E. coli, Bacillus and
23 Pseudomonas amongst others. Preferred eukaryotic hosts
include cells from yeast and fungi, insects, mammals and
25 plants. Even more preferred host cells are plants of the
family Poaceae, and in particular of the genus Lolium,
27 such as Lolium perenne. Accordingly in a preferred
embodiment, the Lol pI gene promoter with a gene encoding
29 a deleterious function positioned relative thereto will be
carried by a recombinant DNA molecule capable of
31 integration into the genome of cells of plants from the
family Poaceae, or more particularly, of the genus Lolium,
33 such as Lolium perenne. Such a recombinant DNA molecule
is transferred to the aforementioned cells by, for

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1 example, electroporation. Ideally, said cells are
2 callus-derived cells. Said callus-derived cells
3 transformed with said recombinant DNA molecule are then
4 permitted to regenerate into whole plants. Whole plants
5 entering the pollen development stage of its life cycle,
6 permit functioning of the Lol pI gene promoter and hence,
7 expression of the gene encoding a deleterious function.
8 Consequently pollen development is inhibited or prevented
9 and a nuclear male sterile plant results therefrom.

11 Alternatively, the Lol pI promoter will direct
12 expression of a gene having advantageous functions, such
13 as a cytokinin. All such recombinant DNA molecules are
14 encompassed by the present invention.

15 The monoclonal antibodies used in the present work
16 to screen the cDNA library for Lol pI clones showed
17 cross-reactivity with allergenic proteins from pollen of
18 various related grass species. This shows there is a
19 homology between allergenic proteins produced by these
20 pollens with Lol pI allergen supporting the applicability
21 of the present invention to all related grasses. For
22 example, this homology can be exploited to isolate DNA
23 encoding other allergenic proteins without the need for
24 protein microsequencing and oligo-nucleotide primers.
25 The present invention also relates to antibodies to
26 recombinant Lol pI and its derivatives, homologues and
27 immunological relatives including its chemical synthetic
28 derivatives. In the following discussion, reference to Lol
29 pI includes its derivatives, homologues and immunological
30 relatives and chemical synthetic derivatives thereof.
31 Such antibodies are contemplated to be useful in
32 developing detection assays (immunoassays) for said Lol pI
33 especially during the monitoring of a therapeutic or
34 diagnostic regimen and in the purification of Lol pI. The
35 antibodies may be

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1 monoclonal or polyclonal. Additionally, it is within the
scope of this invention to include any second antibodies
3 (monoclonal or polyclonal) directed to the first
antibodies discussed above. The present invention further
5 contemplates use of these first or second antibodies in
detection assays and, for example, in monitoring the
7 effect of a diagnostic or an administered pharmaceutical
preparation. Furthermore, it is within the scope of the
9 present invention to include antibodies to the
glycosylated regions of Lol pI (where present), and to any
11 molecules complexed with said Lol pI. Accordingly, an
antibody to Lol pI encompasses antibodies to Lol pI, or
13 antigenic parts thereof, and to any associated molecules
(e.g., glycosylated regions, lipid regions, carrier
15 molecules, fused proteins, and the like).

17 The Lol pI, or parts thereof, considered herein are
purified then utilized in antibody production. Both
19 polyclonal and monoclonal antibodies are obtainable by
immunization with Lol pI, and either type is utilizable
21 for immunoassays. The methods of obtaining both types of
sera are well known in the art. Polyclonal sera are less
23 preferred but are relatively easily prepared by injection
of a suitable laboratory animal with an effective amount
25 of the purified Lol pI, or antigenic parts thereof,
collecting serum from the animal, and isolating specific
27 sera by any of the known immunoadsorbent techniques.
Although antibodies produced by this method are utilizable
29 in virtually any type of immunoassay, they are generally
less favored because of the potential heterogeneity of the
31 produce.

33 The use of monoclonal antibodies in an immunoassay
is particularly preferred because of the ability to

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1 produce them in large quantities and the homogeneity of
the product. The preparation of hybridoma cell lines for
3 monoclonal antibody production derived by fusing an
immortal cell line and lymphocytes sensitized against the
5 immunogenic preparation can be done by techniques which
are well known to those who are skilled in the art. (See,
7 for example, DOUILLARD, and HOFFMAN (1981) and KOHLER and
MILSTEIN (1975; 1976).

9
Unlike preparation of polyclonal sera, the choice of
11 animal is dependent on the availability of appropriate
immortal lines capable of fusing with lymphocytes. Mouse
13 and rat have been the animals of choice in hybridoma
technology and are preferably used. Humans can also be
15 utilized as sources for sensitized lymphocytes if
appropriate immortalized human (or nonhuman) cell lines
17 are available. For the purpose of the present invention,
the animal of choice may be injected with from about
19 0.1 mg to about 20 mg of the purified Lol pI, or parts
thereof. Usually the injecting material is emulsified in
21 Freund's complete adjuvant. Boosting injections may also
be required. The detection of antibody production can be
23 carried out by testing the antisera with appropriately
labelled antigen. Lymphocytes can be obtained by removing
25 the spleen or lymph nodes of sensitized animals in a
sterile fashion and carrying out fusion. Alternatively,
27 lymphocytes can be stimulated or immunized in vitro, as
described, for example, in READING (1982).

29
A number of cell lines suitable for fusion have been
31 developed, and the choice of any particular line for
hybridization protocols is directed by any one of a number
33 of criteria such as speed, uniformity of growth
characteristics, deficiency of its metabolism for a
35 component of the growth medium, and potential for good
fusion frequency.

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1

Intraspecies hybrids, particularly between like strains, work better than interspecies fusions. Several cell lines are available, including mutants selected for the loss of ability to secrete myeloma immunoglobulin.

Cell fusion can be induced either by virus, such as Epstein-Barr or Sendai virus, or polyethylene glycol.

Polyethylene glycol (PEG) is the most efficacious agent for the fusion of mammalian somatic cells. PEG itself may be toxic for cells, and various concentrations should be tested for effects on viability before attempting fusion. The molecular weight range of PEG may be varied from 1000 to 6000. It gives best results when diluted to from about 20% to about 70% (w/w) in saline or serum-free medium.

Exposure to PEG at 37°C for about 30 seconds is preferred in the present case, utilizing murine cells. Extremes of temperature (i.e., about 45°C) are avoided, and preincubation of each component of the fusion system at 37°C prior to fusion can be useful. The ratio between lymphocytes and malignant cells is optimized to avoid cell fusion among spleen cells and a range of from about 1:1 to about 1:10 is commonly used.

The successfully fused cells can be separated from the myeloma line by any technique known by the art. The most common and preferred method is to chose a malignant line which is hyposanthine Guanine Phosphoribosyl Transferase (HGPRT) deficient, which will not grow in an aminopterin-containing medium used to allow only growth of hybrids and aminopterin-containing medium used to allow only growth of hybrids and which is generally composed of hyposanthine $1 \cdot 10^{-4}M$, aminopterin $1 \times 10^{-5}M$, and thymidine $3 \times 10^{-5}M$, commonly known as the HAT medium. The fusion

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1 mixture can be grown in the HAT-containing culture medium
2 immediately after the fusion 24 hours later. The feeding
3 schedules usually entail maintenance in HAT medium for two
4 weeks and then feeding with either regular culture medium
5 or hyposanthine, thymidine-containing medium.

6
7 The growing colonies are then tested for the
8 presence of antibodies that recognize the antigenic
9 preparation. Detection of hybridoma antibodies can be
10 performed using an assay where the antigen is bound to a
11 solid support and allowed to react to hybridoma
12 supernatants containing putative antibodies. The presence
13 of antibodies may be detected by "sandwich" techniques
14 using a variety of indicators. Most of the common methods
15 are sufficiently sensitive for use in the range of
16 antibody concentrations secreted during hybrid growth.

17

Cloning of hybrids can be carried out after 21-23
18 days of cell growth in selected medium. Cloning can be
19 performed by cell limiting dilution in fluid phase or by
20 directly selecting single cells growing in semi-solid
21 agarose. For limiting dilution, cell suspensions are
22 diluted serially to yield a statistical probability of
23 having only one cell per well. For the agarose technique,
24 hybrids are seeded in a semisolid upper layer, over a
25 lower layer containing feeder cells. The colonies from
26 the upper layer may be picked up and eventually
27 transferred to wells.

28
29

Antibody-secreting hybrids can be grown in various
30 tissue culture flasks, yielding supernatants with variable
31 concentrations of antibodies. In order to obtain higher
32 concentrations, hybrids may be transferred into animals to
33 obtain inflammatory ascites. Antibody-containing ascites

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1 can be harvested 8-12 days after intraperitoneal
injection. The ascites contain a higher concentration of
3 antibodies but include both monoclonals and
immunoglobulins from the inflammatory ascites. Antibody
5 purification may then be achieved by, for example,
affinity chromatography.

7

The presence of Lol pI contemplated herein, or
9 antibodies specific for same, in a patient's serum, plant
or mammalian tissue or tissue extract, can be detected
11 utilizing antibodies prepared as above, either monoclonal
or polyclonal, in virtually any type of immunoassay. A
13 wide range of immunoassay techniques are available as can
be seen by reference to U.S. Patent No. 4,016,043,
15 4,424,279 and 4,018,653. This, of course, includes both
single-site and two-site, or "sandwich", assays of the
17 non-competitive types, as well as in the traditional
competitive binding assays. Sandwich assays are among the
19 most useful and commonly used assays and are favoured for
use in the present invention. A number of variations of
21 the sandwich assay technique exist, and all are intended
to be encompassed by the present invention. Briefly, in a
23 typical forward assay, an unlabelled antibody is
immobilized in a solid substrate and the sample to be
25 tested brought into contact with the bound molecule. After
a suitable period of incubation, for a period of time
27 sufficient to allow formation of an antibody-antigen
secondary complex, a second antibody, labelled with a
29 reporter molecule capable of producing a detectable signal
is then added and incubated, allowing time sufficient for
31 the formation of a tertiary complex of
antibody-antigen-labelled antibody (e.g. antibody Lol pI
33 antibody). Any unreacted material is washed away, and the
presence of the antigen is determined by observation of a

- 31 -

1 signal produced by the reporter molecule. The results may
either be qualitative, by simple observation of the
3 visible signal, or may be quantitated by comparing with a
control sample containing known amounts of hapten.
5 Variations on the forward assay include a simultaneous
assay, in which both sample and labelled antibody are
7 added simultaneously to the bound antibody, or a reverse
assay in which the labelled antibody and sample to be
9 tested are first combined, incubated and then added
simultaneously to the bound antibody. These techniques
11 are well known to those skilled in the art, including any
minor variations as will be readily apparent.

13

Although the following discussion is concerned with
15 detecting Lol pI, it is equally applicable to detecting
antibodies to Lol pI and it is intended to be sufficient
17 description thereof. In the typical forward sandwich
assay, a first antibody having specificity for Lol pI, or
19 antigenic parts thereof, contemplated in this invention,
is either covalently or passively bound to a solid
21 surface. The solid surface is typically glass or a
polymer, the most commonly used polymers being cellulose,
23 polyacrylamide, nylon, polystyrene, polyvinyl chloride or
polypropylene. The solid supports may be in the form of
25 tubes, beads, discs of microplates, or any other surface
suitable for conducting an immunoassay. The binding
27 processes are well-known in the art and generally consist
of cross-linking covalently binding or physically
29 adsorbing, the polymer-antibody complex is washed in
preparation for the test sample. An aliquot of the sample
31 to be tested is then added to the solid phase complex and
incubated at 25°C for a period of time sufficient to allow
33 binding of any subunit present in the antibody. The

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1 incubation period will vary but will generally be in the
2 range of about 2-40 minutes. Following the incubation
3 period, the antibody subunit solid phase is washed and
4 dried and incubated with a second antibody specific for a
5 portion of the hapten. The second antibody is linked to a
6 reporter molecule which is used to indicate the binding of
7 the second antibody to the hapten.

8 By "reporter molecule," as used in the present
9 specification, is meant a molecule which, by its chemical
10 nature, provides an analytically identifiable signal which
11 allows the detection of antigen-bound antibody. Detection
12 may be either qualitative or quantitative. The most
13 commonly used reporter molecules in this type of assay are
14 either enzymes, fluorophores or radionuclide containing
15 molecules (i.e. radioisotopes). In the case of an enzyme
16 immunoassay, an enzyme is conjugated to the second
17 antibody, generally by means of glutaraldehyde or
18 periodate. As will be readily recognized, however, a wide
19 variety of different conjugation techniques exist, which
20 are readily available to the skilled artisan. Commonly
21 used enzymes include horseradish peroxidase, glucose
22 oxidase, beta-galactosidase and alkaline phosphatase,
23 amongst others. The substrates to be used with the
24 specific enzymes are generally chosen for the production,
25 upon hydrolysis by the corresponding enzyme, of a
26 detectable color change. For example, p-nitrophenyl
27 phosphate is suitable for use with alkaline phosphatase
28 conjugates; for peroxidase conjugates,
29 1,2-phenylenediamine, 5-aminosalicylic acid, or toluidine
30 are commonly used. It is also possible to employ
31 fluorogenic substrates, which yield a fluorescent product
32 rather than the chromogenic substrates noted above. In
33 all cases, the enzyme-labelled antibody is added to the

- 33 -

1 first antibody hapten complex, allowed to bind, and then
the excess reagent is washed away. A solution containing
3 the appropriate substrate is then added to the tertiary
complex of antibody-antigen-antibody. The substrate will
5 react with the enzyme linked to the second antibody,
giving a qualitative visual signal, which may be further
7 quantitated, usually spectrophotometrically, to give an
indication of the amount of hapten which was present in
9 the sample. "Reporter molecule" also extends to use of
cell agglutination or inhibition of agglutination such as
11 red blood cells on latex beads, and the like.

13 Alternately, fluorescent compounds, such as
florescein and rhodamine, may be chemically coupled to
15 antibodies without altering their binding capacity. When
activated by illumination with light of a particular
17 wavelength, the fluorochrome-labelled antibody adsorbs the
light energy, inducing a state of excitability in the
19 molecule, followed by emission of the light at a
characteristic color visually detectable with a light
21 microscope. As in the EIA, the fluorescent labelled
antibody is allowed to bind to the first antibody-hapten
23 complex. After washing off the unbound reagent, the
remaining tertiary complex is then exposed to the light of
25 the appropriate wavelength, the fluorescence observed
indicates the presence of the hapten of interest.
27 Immunofluorescence and EIA techniques are both very well
established in the art and are particularly preferred for
29 the present method. However, other reporter molecules,
such as radioisotope, chemiluminescent or bioluminescent
31 molecules, may also be employed. It will be readily
apparent to the skilled technician how to vary the
33 procedure to suit the required purpose. It will also be
apparent that the foregoing can be used to detect directly
35 or indirectly (i.e., via antibodies) the Lol pI of this
invention.

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1

Accordingly, one aspect of the present invention
3 contemplates a method of detecting Lol pI or a derivative
or homologue thereof or a allergenic protein
5 immunologically reactive with said Lol pI or its
derivative or homologue in serum, tissue extract, plant
7 extract or other biologically fluid comprising the steps
of contacting said serum, extract or fluid to be tested
9 with an antibody to Lol pI for a time and under conditions
sufficient for an allergenic protein-antibody complex to
11 form and subjecting said complex to a detecting means.

The present invention also contemplates a method of
13 detecting an antibody to an allergenic protein from pollen
of the family Poaceae (Gramineae) in serum or other
15 biological fluid comprising contacting said serum or fluid
with recombinant Lol pI or its antigenic derivative for a
17 time and under conditions sufficient for an antibody - Lol
pI complex to form and subjecting said complex to a
19 detecting means. The latter complex may be detected by
the Lol pI having attached thereto a reporter molecule or
21 by addition of a second antibody labelled with a reporter
molecule.

23

Accordingly, the present invention is also directed
25 to a kit for the rapid and convenient assay for antibodies
to Lol pI or its derivatives, homologues or immunological
27 relatives in mammalian body fluids (e.g. serum, tissue
extracts, tissue fluids), in vitro cell culture
29 supernatants, and cell lysates. The kit is
compartmentalized to receive a first container adapted to
31 contain recombinant Lol pI, or to an antigenic component
thereof, and a second container adapted to contain an
33 antibody to Lol pI said antibody being labelled with a
reporter molecule capable of giving a detectable signal as

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1 hereinbefore described. If the reporter molecule is an
enzyme, then a third container adapted to contain a
3 substrate for said enzyme is provided. In an exemplified
use of the subject kit, a sample to be tested is contacted
5 tot he contents of the first container for a time and
under conditions for an antibody, if present, to bind to
7 Lol pI in said first container. If Lol pI of the first
container has bound to antibodies in the test fluid, the
9 antibodies of the second container will bind to the
secondary complex to form a tertiary complex and, since
11 these antibodies are labelled with a reporter molecule,
when subjected to a detecting means, the tertiary complex
13 is detected. Therefore, one aspect of the present
invention is a kit for the detection of antibodies to a
15 protein having allergenic properties, said protein from
pollen of the family Poaceae (Gramineae), the kit being
17 compartmentalized to receive a first container adapted to
contain recombinant Lol pI or its antigenic derivative or
19 homologue, and a second container adapted to contain and
antibody to Lol pI or its derivative or homologue, said
21 antibody labelled with a reporter molecule capable of
giving a detectable signal. The "reporter molecule" may
23 also involve agglutination of red blood cells (RBC) on
latex beads. In this kit the reporter molecule is a
25 radioisotope, an enzyme, a fluorescent molecule, a
chemiluminescent molecule, bioluminescent molecule or
27 RBC. The kit alternatively comprises a container adapted
to contain recombinant Lol pI or is antigenic derivative
29 or homologue labelled with a reporter molecule capable of
giving a detectable signal.

31

Because of the presence of allergens in the
33 environment, hayfever and seasonal asthma continue to have
significant morbidity and socio-economic impact on Western

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1 communities, despite advances made in their pharmacology
and immunology. While the available spectrum of drugs,
3 including anti-histamines and steroids have resulted in
spectacular improvement in the treatment of allergic
5 disease, yet they have unfortunate side-effects associated
with longterm usage. Because of these problems, renewed
7 interest has been shown in the immunotherapy of allergic
disease. Immunotherapy involves the injection of potent
9 allergen extracts to desensitize patients against allergic
reactions (BOUSQUET, & MICHEL, 1989.) Unfortunately, the
11 pollen preparations used as allergens are polyvalent and
of poor quality. Consequently, concentrations used are
13 frequently high in order to induce IgG responses, but may
be lethal through triggering of systemic reactions,
15 including anaphylaxis. The cloned gene product or
synthetic peptides based on the sequence of allergens
17 provides a safer medium for therapy since it can be
quality controlled, characterized and standardized.

19

The precise mechanism for symptomatic relief remains
21 hypothetical. It is established that desensitization
therapy induces the formation of allergen-specific
23 non-mast cell-binding IgG which blocks the combination of
mast cell-bound IgE and allergen. This prevents mediator
25 release, and triggering of the allergic response. Recent
studies of ragweed pollen sensitivity showed that there is
27 a correlation between allergen-specific IgG levels and
relief from allergic symptoms (Lichtenstein et al.,
29 1983). Application of reagents which can trigger
allergen-specific IgG production during immunotherapy
31 could significantly enhance the success rate of this
treatment.

33

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1 Currently immunotherapy is one of the most
frequently administered treatments in allergology, and in
3 USA it is considered the first choice. Advantages of this
treatment for pollen rhinitis is that treatment takes up
5 to 3 years, while pharmacotherapy must be carried out
during the patient's entire life time. Patients given
7 pollen extract for immunotherapy showed a clinical benefit
that lasted for four years after the end of treatment
9 (GRAMMER et al., 1984.

11 Accordingly, Lol pI, its derivatives, homologues or
immunological relatives is useful in developing a vaccine
13 to desensitized humans to allergies due to grass pollen.

15 Accordingly, the present invention contemplates a
method for desensitizing a human allergic to grass pollen
17 which comprises administering to said human a
desensitizing-effective amount of Lol pI or a derivative,
19 homologue, or immunological relative thereof whether made
by recombinant or synthetic means for a time and under
21 conditions sufficient to effect desensitization of said
human to said grass pollen.

23

The present invention, therefore, contemplates a
25 pharmaceutical composition comprising a desensitizing
effective amount of Lol pI or its derivatives, homologues
27 or immunological relatives and a pharmaceutically
acceptable carrier. The active ingredients of a
29 pharmaceutical composition comprising Lol pI or the like
are contemplated to exhibit excellent therapeutic
31 activity, for example, in the desensitization of humans
allergic to grass pollen when administered in amount

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1 which depends on the particular case. For example, from
2 about 0.5 ug to about 20 mg per kilogram of body weight
3 per day may be administered. Dosage regima may be
4 adjusted to provide the optimum therapeutic response. For
5 example, several divided doses may be administered daily
6 or the dose may be proportionally reduced as indicated by
7 the exigencies of the therapeutic situation. The active
8 compound may be administered in a convenient manner such
9 as by the oral, intravenous (where water soluble),
10 intramuscular, subcutaneous, intranasal, intradermal or
11 suppository routes or implanting (eg using slow release
12 molecules). Depending on the route of administration, the
13 active ingredients which comprise Lol pI or the like may
14 be required to be coated in a material to protect said
15 ingredients from the action of enzymes, acids and other
16 natural conditions which may inactivate said ingredients.
17 For example, the low lipophilicity of Lol pI or the like
18 will allow it to be destroyed in the gastrointestinal
19 tract by enzymes capable of cleaving peptide bonds and in
20 the stomach by acid hydrolysis. In order to administer
21 Lol pI or the like by other than parenteral
22 administration, they will be coated by, or administered
23 with, a material to prevent its inactivation. For
24 example, Lol pI or the like may be administered in an
25 adjuvant, co-administered with enzyme inhibitors or in
26 liposomes. Adjuvant is used in its broadest
27 sense and includes any immune stimulating
28 compound such as interferon. Adjuvants
29 contemplated herein include resorcinols, non-ionic
30 surfactants such as polyoxyethylene oleyl ether and
31 n-hexadecyl polyethylene ether. Enzyme inhibitors include
32 pancreatic trypsin inhibitor, diisopropylfluorophosphate
33 (DEP) and trasylol. Liposomes include
34 water-in-oil-in-water CGF emulsions as well as
35 conventional liposomes.

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1 The active compounds may also be administered
parenterally or intraperitoneally. Dispersions can also
3 be prepared in glycerol, liquid polyethylene glycols, and
mixtures thereof and in oils. Under ordinary conditions
5 of storage and use, these preparations contain a
preservative to prevent the growth of microorganisms.

7

 The pharmaceutical forms suitable for injectable use
9 include sterile aqueous solutions (where water soluble) or
dispersions and sterile powders for the extemporaneous
11 preparation of sterile injectable solutions or
dispersion. In all cases the form must be sterile and
13 must be fluid to the extent that easy syringability
exists. It must be stable under the conditions of
15 manufacture and storage and must be preserved against the
contaminating action of microorganisms such as bacteria
17 and fungi. The carrier can be a solvent or dispersion
medium containing, for example, water, ethanol, polyol
19 (for example, glycerol, propylene glycol, and liquid
polyethylene glycol, and the like), suitable mixtures
21 thereof, and vegetable oils. The proper fluidity can be
maintained, for example, by the use of a coating such as
23 lecithin, by the maintenance of the required particle size
in the case of dispersion and by the use of
25 surfactants. The preventions of the action of
microorganisms can be brought about by various
27 antibacterial and antifungal agents, for example,
parabens, chlorobutanol, phenol, sorbic acid, thimerosal,
29 and the like. In many cases, it will be preferable to
include isotonic agents, for example, sugars or sodium
31 chloride. Prolonged absorption of the injectable
compositions can be brought about by the use in the
33 compositions of agents delaying absorption, for example,
aluminum monostearate and gelatin.

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1

2 Sterile injectable solutions are prepared by
3 incorporating the active compounds in the required amount
4 in the appropriate solvent with various of the other
5 ingredients enumerated above, as required, followed by
6 filtered sterilization. Generally, dispersions are
7 prepared by incorporating the various sterilized active
8 ingredient into a sterile vehicle which contains the basic
9 dispersion medium and the required other ingredients from
10 those enumerated above. In the case of sterile powders
11 for the preparation of sterile injectable solutions, the
12 preferred methods of preparation are vacuum drying and the
13 freeze-drying technique which yield a powder of the active
14 ingredient plus any additional desired ingredient from
15 previously sterile-filtered solution thereof.

17 When Lol pI or the like is suitably protected as
18 described above, the active, compound may be orally
19 administered, for example, with an inert diluent or with
20 an assimilable edible carrier, or it may be enclosed in
21 hard or soft shell gelatin capsule, or it may be
22 compressed into tablets, or it may be incorporated
23 directly with the food of the diet. For oral therapeutic
24 administration, the active compound may be incorporated
25 with excipients and used in the form of ingestible
26 tablets, buccal tablets, troches, capsules, elixirs,
27 suspensions, syrups, wafers, and the like. Such
28 compositions and preparations should contain at least 1%
29 by weight of active compound. The percentage of the
30 compositions and preparations may, of course, be varied
31 and may conveniently be between about 5 to about 80% of
32 the weight of the unit. The amount of active compound in
33 such therapeutically useful compositions in such that a
34 suitable dosage will be obtained. Preferred compositions
35 or preparations

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1 according to the present invention are prepared so that an
oral dosage unit form contains between about 10 ug and
3 2000 mg of active compound.

5 The tablets, troches, pills, capsules and the like
may also contain the following: A binder such as gum
7 gragacanth, acacia, corn starch or gelatin; excipients
such as dicalcium phosphate; a disintegrating agent such
9 as corn starch, potato starch, alginic acid and the like;
a lubricant such as magnesium stearate; and a sweetening
11 agent such a sucrose, lactose or saccharin may be added or
a flavoring agent such as peppermint, oil of wintergree,
13 or cherry flavouring. When the dosage unit form is a
capsule, it may contain, in addition to materials of the
15 above type, a liquid carrier. Various other materials may
be present as coatings or to otherwise modify the physical
17 form of the dosage unit. For instance, tablets, pills, or
capsules may be coated with shellac, sugar or both. A
19 syrup or elixir may contain the active compound, sucrose
as a sweetening agent, methyl and propylparabens as
21 preservatives, a dye and flavoring such as cherry or
orange flavor. Of course, any material used in preparing
23 any dosage unit form should be pharmaceutically pure and
substantially non-toxic in the amounts employed. In
25 addition, the active compound may be incorporated into
sustained-release preparations and formulations.

27

As used herein "pharmaceutically acceptable carrier"
29 includes any and all solvents, dispersion media, coatings,
antibacterial and antifungal agents, isotonic and
31 absorption delaying agents, and the like. The use of such
media and agents for pharmaceutical active substances is
33 well known in the art. Except insofar as any conventional
media or agent is incompatible with the active ingredient,

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1 use thereof in the therapeutic compositions is
2 contemplated. Supplementary active ingredients can also
3 be incorporated into the compositions.

5 It is especially advantageous to formulate
6 parenteral compositions in dosage unit form for ease of
7 administration and uniformity of dosage. Dosage unit form
8 as used herein refers to physically discrete units suited
9 as unitary dosages for the mammalian subjects to be
10 treated; each unit containing a predetermined quantity of
11 active material calculated to produce the desired
12 therapeutic effect in association with the required
13 pharmaceutical carrier. The specification for the novel
14 dosage unit forms of the invention are dictated by and
15 directly dependent on (a) the unique characteristics of
16 the active material and the particular therapeutic effect
17 to be achieved, and (b) the limitations inherent in the
18 art of compounding such an active material for the
19 treatment of disease in living subjects having a diseased
20 condition in which bodily health is impaired as herein
21 disclosed in detail.

23 The principal active ingredient is compounded for
24 convenient and effective administration in effective
25 amounts with a suitable pharmaceutically acceptable
26 carrier in dosage unit form as hereinbefore disclosed. A
27 unit dosage form can, for example, contain the principal
28 active compound in amounts ranging from 0.5 μ g to about
29 2000 mg. Expressed in proportions, the active compound is
30 generally present in from about 0.5 μ g to about 2000 mg/ml
31 of carrier. In the case of compositions containing
32 supplementary active ingredients, the dosages are
33 determined by reference to the usual dose and manner of
administration of the said ingredients.

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1

The present invention is further illustrated by the
3 following non-limiting examples.

5 EXAMPLE 1

Extraction of RNA

7

4g of fresh ryegrass pollen (collected from field
9 sources near Melbourne and stored under liquid N₂) was
suspended in 10 ml of extraction buffer (50mM Tris buffer,
11 pH9, 0.2M NaCl, 10mM Mg acetate), containing vanadyl
ribonucleoside complexes to 10mM (BERGER AND BIRKENMEIER,
13 1979) and DEPC to 0.1%. The pollen sluchs was ground in a
mortar and pestle under liquid N₂ for 10-20 min to provide
15 a homogenate in which all pollen grains are broken. The
slurry was transferred to Nalgene centrifuge tubes with 1%
17 (w/v) SDS, 10mM EDTA and 0.5% (w/v) N-lauroyl sarcosine.
An equal volume of warm, high grade buffered phenol (from
19 IBI), treated with 0.1% (w/v) hydroxyquinoline (MANIATIS
et al., 1982) was added, and the mixture shaken for 10
21 min. An equivalent volume of 24:1 parts
chloroform:isoamylalcohol was added and shaking
23 continued. Tubes were centrifuged at 15,000 rpm for 20
min at 10°C to separate the phases and remove the
25 insoluble material and cell debris. The aqueous phase was
reextracted with P:C:I four times until the phenol phase
27 remained clear, (with phase separation at 2,500 rpm for 15
min at room temperature), and the aqueous phase was
29 transferred to Corex centrifuge tubes. 2.5 volumes of
100% (v/v) ethanol were added and the solution mixed by
31 pipette, and allowed to precipitate overnight at -20°C,
and spun at 15,000 rpm for 20 min at 0°C. The pellet was
33 resuspended in 10 ml of EDTA to remove the
vanadyl-ribonucleoside complexes, and LiCl added to give

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1 a final concentration of 2M. The solution was kept at 0°C
overnight and centrifuged at 15,000 rpm for 30 min at
3 4°C. The pellet was washed with cold 2M LiCl and 5mM EDTA
(pH 7.3), the liquid poured off, and the pellet
5 resuspended in 1 ml of water. The solution is heated to
65°C, and 0.1 ml of 3M Na- acetate and 2.2 ml of ethanol
7 were added for overnight precipitation of total RNA at
-20°C. The pellet was washed gently with 70% (v/v)
9 ethanol vacuum-dried, and resuspended in 0.5 ml water.
The suspension was stored at -70°C until required for poly
11 (A+) RNA selection.

13 One gram of ryegrass pollen contained 1 mg total
RNA. Poly (A+) mRNA was selected by affinity
15 chromatography on Poly (U)-Sephadex (Pharmacia)
according to standard methods. The integrity of poly (A+)
17 mRNA was examined in terms of its ability to act as a
template for synthesis of single-stranded cDNA as well as
19 its translational activity in the rabbit reticulocyte
system.

21

EXAMPLE 2

23 Preparation of cDNA clones

25 Synthesis of first strand cDNA was from 5ug (poly
A+) RNA in 50 ul reaction buffer (50mM Tris buffer, pH
27 8.3, containing 40mM KCl, 10mM MgCl₂, 5mM DTT, 1mM each of
dATP, dGTP, dTTP, and dCTP, 50 units of human placental
29 ribonuclease inhibitor (HPRI), 5 ug of
oligodeoxythymidylic acid primer, 80 uCi of [Alpha-³²P]
31 dCTP (3000Ci/mmol; Amersham) and 100 units of reverse
transcriptase. The mixture was incubated for 60 min at
33 42°C. Second strand cDNA was synthesized using the
mRNA/cDNA hybrids as substrate, 4 units of E. coli DNA

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1 ribonuclease H to produce nicks in the mRNA template, and
2 115 units of E. coli DNA polymerase I to catalyse the
3 replacement of the mRNA strand by DNA. The reaction
4 mixture was incubated sequentially at 12°C for 60 min
5 each, and the reaction stopped by heating at 70°C for 10
6 min. 10 units of T4 DNA polymerase were added to remove
7 small 3' overhangs from the first strand cDNA (GUBLER AND
8 HOFFMAN, 1983). The reaction was stopped by adding one
9 tenth volume of 20mM EDTA and 1% (w/v) SDS. The double
10 strand (ds) cDNA was purified by phenol/chloroform
11 extraction followed by precipitation with ethanol.

13 In order to construct a lambda-gt 11 cDNA expression
14 library, 500 ng of double stranded cDNA was incubated with
15 20 units of EcoRI methylase at 37°C for 60 min.

17 1 µg of phosphorylated EcoRI linkers (5'-d[pGGAATTCC])
18 was ligated to the double stranded cDNA in ligation buffer
19 with 5 units of T4 DNA ligase at 15°C overnight. The
20 EcoRI-linkered cDNA was digested with 100 units of EcoRI
21 linkers through a Sephacryl column.

23 A 50ng of linkered cDNA was ligated to 1 µg of
24 dephosphorylated EcoRI-cut lambda-gt 11 DNA (Promega) with
25 2.5 units of T4 DNA ligase for 20 h at 15°C in a total
26 volume of 10 µl. The ligated lambda-gt 11 DNA was
27 precipitated with 30 mM Na-acetate and 2.7 volumes of
28 ethanol at -70°C for 2h. The lambda-gt 11 DNA ligated to
29 cDNA was packaged in vitro at 20°C for 2 h using 25 µl of
30 the lambda packaging mixture (Promega). The cDNA library
31 was titrated on E. coli strain Y1090r on plates containing
32 1mg/ml X-gal, and 0.4 mg/ml IPTG. The cDNA was amplified
33 as plate lysate on E. coli strain Y1090r⁻ at a density of
15,000 plaques per 150mm plate.

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1

EXAMPLE 3

3 Screening the lambda-gt 11 cDNA library using specific
monoclonal antibody probes.

5

Ryegrass pollen allergen-specific monoclonal
7 antibodies were developed and characterized by SMART
et al. (1983). Sera from patients allergic to ryegrass
9 pollen were kindly provided by Dr David Hill from the
Royal Children's Hospital, Melbourne.

11

The following procedure for screening the lambda-gt
13 11 expression library is a modification of a previously
described method (HUYNH, YOUNG AND DAVIS, 1985). A single
15 colony of E. coli Y1090r⁻ was grown at 37°C with good
aeration to OD600 of 0.7 - 0.9, in LB medium containing
17 100 ug/ml ampicillin and 0.4% (w/v) maltose. The cells
were pelleted and resuspended in 10 mM MgSO₄ in 40% of
19 the culture volume. The E. coli Y1090r⁻ cells (0.3 ml)
were then infected with approximately 18,000 - 20,000
21 recombinant phage at 37°C for 15 minutes, plated onto
150mB LB plates in 0.7% (w/v) agarose and incubated at
23 42°C for 3 hours. The plates were overlaid with dry
132mm nitrocellulose filters presoaked in 10mIPTG then
25 incubated for 6 h at 37°C and the filters removed. A
second IPTG-treated filter was placed on the bacterial
27 lawn and the plates incubated overnight at 37°C. Filter
plaque lifts were dried at room temperature, and washed
29 with TBS for 10 minutes. The TBS was removed and 10ml of
TBS containing 10% (w/v) non-fat milk powder was added and
31 the filters were gently agitated for 1 h, drained, rinsed
for 30 sec with TBS then washed for 10 minutes with TBS
33 plus 0.1 (v/v) Tween -20 followed by two more washes of
TBS for 10 min each. The filters were incubated for 3 h in

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1 TBS containing 2% (w/v) BSA and monoclonal antibodies
(ascites) to ryetrass pollen allergens at a dilution of
3 1:500 with gentle agitation. Following washing in
TBS/0.1% (v/v) Tween-20, the filters were incubated ro 1.5
5 h in TBS containing 2% (w/v) BSA and peroxidase-conjugated
affinity - purified anti-mouse IgG at a dilution of
7 1:500. The filters were washed and developed using fresh
chromogenic peroxidase substrate 4-chloro-1-naphthol, 60mg
9 dissolved in 20ml ice-cold methanol, and 80ml TBS
containing 0.03% (v/v) H₂O₂. For each filter, 10ml of
11 developing solution was used. After purple spots appeared
on the filters, the developing solution was removed and
13 the filters washed with distilled water to stop the
reaction.

15

The developed filter was used to locate specific
17 plaque areas on the plate, corresponding to a positive
signal. Positive phage plaques were lifted from the
19 plates a sagarose plugs and the eluted phage purified to
individual antigen-positive lambda-gt 11 clones by
21 rescreening at lower density on 85mm petri dishes with
82mm nitrocellulose circles. Once plaque purification had
23 been achieved, each of the lambda-gt 11 clones bearing
allergen cDNA was lated at low denisty, and duplicate
25 filter lifts were made. The ability of the recombinanat
allergen to bind with antisera from allergic patients was
27 detected using the same procudeure as described above,
except that the overnight filter lift was incubated in the
29 allergic antisera at a dilution of 1:10 whereas the first
lift was treated with monoclonal antibodies.

31

EXAMPLE 4

33 Preparation of recombinant allergenic proteins from
lambda-gt 11 recombinant lysogens and Western Blot
35 Analysis.

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1

Bacteria (E. coli strain Y1089) to be lysogenized by the recombinant phage were grown to saturation in LB medium (pH 7.5) containing 0.2% (w.v) maltose at 37°C. One ml of the cells was collected by centrifugation and resuspended in 300 ul of LB medium containing 10mM MgCl₂. The cells (approximately 1×10^8 cells) were infected with about 1×10^9 pfu of lambda-gt 11 recombinant phage containing cDNA inserts coding for the allergenic proteins (e.g. clone 12R and clone 6R) at 32°C for 20 minutes. The infected cells were serially diluted and plated at the density of 100-200 colonies per plate and incubated overnight at 32°C. Individual colonies were spotted onto replicate LB plates, of which one was incubated at 42°C, and the other at 32°C overnight. Recombinant lysogen clones were indicated by growth at 32°C but not at 42°C, and occurred at a frequency of 20% for clone 12R, and 3-4% for clone 6R.

19

In order to obtain a preparative amount of the recombinant allergenic proteins, a single lysogen colony of Y1089 was inoculated into 10ml of LB medkium and incubated 32°C with good aeration until the OD600 reached 0.5. The culture was quickly shifted to a 42°C water bath and incubated for 20 minutes with shaking.

The lac operon repressor was inactivated by addition of 100 ul of 1M IPTG. The culture was then incubated at 37°C for 1 hour allowing the lac Z gene to be expressed and the allergenic proteins to be synthesized as a fusion protein with beta-galactosidase. Cells were harvested by spinning at 3000 rpm for 10 minutes at room temperature, resuspended in 150 ul of Sample buffer and immediately frozen in liquid nitrogen. The cells were

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1 lysed by thawing at room temperature. For electrophoretic
analysis of proteins, 150 ul of SDS sample buffer
3 containing bromophenol blue tracking dye was added to the
freeze-thaw lysate. Samples were boiled for 3 minutes and
5 the insoluble material removed by micro-entrifugation for
3 minutes.

7

Proteins were resolved by 7-10% (w/v) SDS -
9 polyacrylamide gel electrophoresis and visualised by
Coomassie Blue-staining with duplicate samples . .
11 electroblotted onto nitrocellulose filter using the
Bio-Rad Trans Blot apparatus (0.15 amps overnight).
13 Fusion proteins were detected with monoclonal antibodies
and visualized using the screening procedure described
15 previously.

17 EXAMPLE 5

Northern analysis.

19

Total RNA was extracted from pollen, leaf, hydrated
21 seed and root samples as previously described for pollen,
and 20 ug RNA/sample electrophoresed in formaldehyde/1.2%
23 (w/v) agarose gels (MANIATIS et al., 1982) run at 70V for
4 hrs in running buffer containing 20mM morpholinopropane
25 sulphonic acid, 5mM sodium acetate and 0.1mM EDTA, to pH
7.0. The RNA's were transferred to nitrocellulose (Hybond
27 C) filters and pre-equilibrated 2 hours at 50°C in
hybridization buffer containing 50% (v/v) deionised
29 formamide, 2X SSPE, 7 % (w/v) SDS, 0.5% (w/v) non-fat milk
powder, 1% PEG 20,000, and 0.5mg/ml non-homologous herring
31 sperm carrier DNA. Fresh hybridization buffer containing
the random primed Lol pI DNA probe was added and incubated
33 at 50°C for overnight hybridization. Filters were washed
vigorously in 2X SSC, 0.1% (w/v) SDS for 15 minutes at RT,

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1 then 0.5 X SSC, 1% (w/v) SDS at 50°C for 15 minutes,
followed by a brief rinse in 0.5x SSC, 0.1% (w/v) SDS,
3 blotted lightly and wrapped in Glad Wrap. Kodak film was
exposed for 18 hours at -70°C.

5

EXAMPLE 6

7 Expression of Lol pI cDNA products reacted with IgE from
allergic sera.

9

The cDNA insert from lambda-gt 11-12R which codes
11 for Lol pI was sub-cloned into the EcoRI site of the
plasmid expression vector pGEX where it can be expressed
13 as a fusion protein with glutathione transferase. E. coli
infected with this plasmid pGEX-12R or with the
15 non-recombinant vector alone, were grown at a log phase
culture, and the bacteria pelleted by centrifugation.
17 These bacteria were lysed and the total proteins separated
on SDS-PAGE gel. A western blot shows that only bacteria
19 containing recombinant-plasmids possess a protein
component reactive with specific IgE in sera taken from
21 donors known to be allergic to ryegrass pollen. Those
results are shown in Figure 6.

23

EXAMPLE 7

25 Cross-reactivity of Lol pI with homologous allergens from
other grass pollen.

27

Lol pI is a protein of MW 34 kD, and SDS-PAGE shows
29 that other common grasses possess a homologous protein of
similar molecular weight. Our results show that these
31 proteins share a common antigenic epitope (detected by
monoclonal antibodies), and are allergens in terms of
33 specific IgE- binding. Results are shown in Figure 7.
Because of this allergenic similarity, Lol pI is the

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1 immunodominant allergen of grass pollen. A consequence is
that the cDNA clone 12R can be used as a heterologous
3 probe to isolate the homologous cDNA clones for allergens
from other grass pollens.

5

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- 56 -

CLAIMS

1. A recombinant vector comprising a DNA sequence encoding a protein displaying allergenic activity from pollen of a grass species.
2. The recombinant vector according to claim 1, wherein the allergenic protein is from pollen of grass belonging to the family Poaceae (Gramineae).
3. The recombinant vector according to claim 2, wherein the allergenic protein is from pollen of grass belonging to the genus Lolium.

4. The recombinant vector according to claim 3, wherein the allergenic protein is immunologically cross-reactive with antibody to Lol pI protein of Lolium perenne pollen, namely:

Pooid (festucoid) grasses. Group 1: Triticanae: Bromus inermis, smooth brome; Agropyron repens, English couch; A. cristatum; Secale cereale, rye; Triticum aestivum, wheat. Group 2: Poanae: Dactylis glomerata, orchard grass or cocksfoot; Festuca elatior, meadow fescue; Lolium perenne, perennial ryegrass; L. multiflorum, Italian ryegrass; Poa pratensis, Kentucky bluegrass; P. compressa, flattened meadow grass; Avena sativa, oat; Holcus lanatus, velvet grass or Yorkshire fog; Anthoxanthum odoratum, sweet vernal grass; Arrhenatherum elatius, oat grass; Agrostis alba, red top; Phleum pratense, timothy; Phalaris arundinacea, reed canary grass.

Panicoid grass, Paspalum notatum, Bahia grass, Andropogonoid grasses: Sorghum halepensis, Johnson grass; Zea mays, maize.

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5. The recombinant vector according to claim 4 wherein the allergenic protein is Lol pI of ryegrass, Lolium perenne, pollen, or a derivative or homologue thereof.
6. The recombinant vector according to claim 5 comprising a DNA sequence as depicted in Figure 5 or a degenerate or homologous form thereof.
7. A recombinant DNA molecule comprising a eukaryotic or prokaryotic origin of replication, a detectable marker, a DNA sequence encoding the Lol pI allergenic protein or a derivative or a homologue thereof or an allergenic protein cross-reactive with an antibody to said Lol pI protein or its derivatives or homologues and optionally a promoter sequence capable of directing transcription of said DNA sequence.
8. The recombinant DNA molecule according to claim 7 comprising a DNA sequence as depicted in Figure 5 or a degenerate or homologous form thereof.
9. The recombinant DNA molecule according to claim 7 or 8, wherein the promoter is the Lol pI gene promoter.
10. A host cell carrying a vector or recombinant DNA molecule according to anyone of claims 1 to 9.
11. A method for isolating and identifying DNA encoding an allergenic protein of pollen from the family Poaceae (Gramineae) comprising screening by hybridization DNA isolated from said family with a DNA or RNA sequence encoding Lol pI protein of Lolium perenne pollen or its derivatives or homologues .

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12. The method according to claim 11, wherein the DNA to be identified comprises DNA from a cDNA library, which is prepared by reverse transcription on a template of mRNA of grass pollen showing allergenic activity.

13. A method of producing recombinant Lol pI or a derivative or homologue thereof or an allergenic protein immunologically reactive to antibodies to Lol pI or a derivative or homologue thereof, comprising culturing an organism containing a replicable recombinant DNA molecule, said molecule comprising a promoter capable of expression in said organism, the gene encoding Lol pI or its derivative or homologue or an immunologically related protein of Lol pI located downstream of and transcribed from said promoter, a selectable marker and a DNA vehicle containing a prokaryotic or eukaryotic origin of replication, under conditions and for a time sufficient for said recombinant DNA molecule to be stably maintained and direct the synthesis of Lol pI or its derivative, homologue or immunological relative and then isolating same.

14. The method according to claim 15, wherein the promoter is the Lol pI promoter or homologue or degenerate form thereof and the host organism is one in which said promoter will function.

15. Non-native Lol pI or a derivative or homologue thereof or a non-native allergenic protein immunologically reactive to antibodies to said Lol pI or its derivative or homologue.

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16. An antibody to non-native Lol pI or a derivative or homologue thereof or to a non-native allergenic protein immunologically reactive to antibodies to said Lol pI or its derivative or homologue.

17. A method of detecting Lol pI or a derivative or homologue thereof or an allergenic protein immunologically reactive with said Lol pI or its derivative or homologue in serum, tissue extract, plant extract or other biological fluid comprising the steps of contacting said serum, extract or fluid to be tested with an antibody according to claim 14 for a time and under conditions sufficient for an allergenic protein-antibody complex to form and subjecting said complex to a detecting means.

18. The method according to claim 17, wherein the antibody is labelled with a material providing a detectable signal, said material selected from the group consisting of a radioactive isotope, and enzyme, a fluorescent molecule, a chemiluminescent molecule, a bioluminescent molecule or a cell.

19. The method according to claim 17, wherein the allergenic protein-antibody complex is detected by contacting said complex with a second antibody specific to the first antibody, said second antibody labelled with a material providing a detectable signal, said material selected from the group consisting of a radioactive isotope, an enzyme, a fluorescent molecule, a chemiluminescent molecule, a bioluminescent molecule or a cell for a time and under conditions sufficient for a tertiary complex to form and then detecting said signal.

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20. A method of detecting an antibody to an allergenic protein from pollen of the family Poaceae (Gramineae) in serum or other biological fluid comprising contacting said serum or fluid with recombinant Lol pI or its antigenic derivative for a time and under conditions sufficient for an antibody - Lol pI complex to form and subjecting said complex to a detecting means.

21. The method according to claim 20, wherein the recombinant Lol pI or its antigenic derivative is optionally labelled with a reporter molecule.

22. The method according to claim 20, wherein the complex is detected by contacting said complex with a second antibody specific to said Lol pI or its antigenic derivative and said second antibody being labelled with a reporter molecule, for a time and under conditions sufficient for a tertiary complex to form and then detecting said reporter molecule.

23. The method according to claim 21 or 22 wherein said reporter molecule is selected from the group consisting of a radioactive isotope, and enzyme, a fluorescent molecule, a chemiluminescent molecule a bioluminescent molecule or a cell.

24. A kit for the detection of antibodies to a protein having allergenic properties, said protein from pollen of the family Poaceae (Gramineae), the kit being compartmentalized to receive a first container adapted to contain recombinant Lol pI or its antigenic derivative or homologue, and a second container adapted to contain an antibody to Lol pI or its derivative or homologue, said antibody labelled with a reporter molecule capable of giving a detectable signal.

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25. The kit according to claim 24, wherein the reporter molecule is a radioisotope, an enzyme, a fluorescent molecule, a chemiluminescent molecule, a bioluminescent molecule or a cell.
26. The kit according to claim 25, wherein the reporter molecule is an enzyme.
27. The kit according to claim 26, wherein the kit further comprises a third container adapted to contain a substrate for the enzyme.
28. The kit according to claim 24 alternatively comprising a container adapted to contain recombinant Lol pI or is antigenic derivative or homologue labelled with a reporter molecule capable of giving a detectable signal.
29. A recombinant DNA molecule comprising a ryegrass pollen promoter sequence or homologue or degenerate form thereof located on said molecule and further having one or more restriction endonuclease sites downstream of said promoter such that a nucleotide sequence inserted into one or more of these sites is transcribeable in the correct reading frame.
30. The recombinant DNA molecule according to claim 29, wherein said promoter is the Lol pI gene promoter.
31. The recombinant DNA molecule according to claim 30 further comprising a selectable marker.
32. The recombinant molecule according to claim 29 or 30 or 31 further comprising means for stable inheritance in a prokaryotic and/or eukaryotic cell.

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33. The recombinant DNA molecule according to claim 32, wherein said means comprises a prokaryotic or eukaryotic origin of replication thereby permitting said molecule to replicate extrachromosomally in a host cell.

34. The recombinant DNA molecule according to claim 33, wherein said molecule is replicable in prokaryotic cells.

35. The recombinant DNA molecule according to claim 34, wherein the prokaryotic cells comprise Escherichia coli, Pseudomonas or Bacillus.

36. The recombinant DNA molecule according to claim 33, wherein said molecule is replicable in eukaryotic cells.

37. The recombinant DNA molecule according to claim 36, wherein the eukaryotic cells comprise cells from yeast, insects, mammals or plants.

38. The recombinant DNA molecule according to claim 37, wherein the eukaryotic cells are plant cells derived from the family Poaceae.

39. The recombinant DNA molecule according to claim 32, wherein said molecule replicates by insertion into the genome of a host cell and replicates in synchrony with said genome.

40. The recombinant DNA molecule according to any one of claims 29 to 39 further comprising a nucleotide sequence encoding a polypeptide or portion thereof or a mRNA or a portion thereof inserted into one of the restriction endonuclease sites downstream of said promoter such that said nucleotide sequence is transcribeable in the correct reading frame.

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41. The recombinant DNA molecule according to claim 40, wherein said nucleotide sequence encodes an allergenic protein, a cytokinin or a protein having a deleterious function on a plant cell, or their derivatives.

42. The recombinant DNA molecule according to claim 41, wherein the allergenic protein in Lol pI or its derivative.

43. The recombinant DNA molecule according to claim 41, wherein the nucleotide sequence encodes a toxin, said toxin active against cells derived from the family Poaceae.

44. The recombinant DNA molecule according to claim 40, wherein the nucleotide sequence encodes an antisense RNA capable of inhibiting translation of a gene in a cell from the family Poaceae.

45. A prokaryote or eukaryote transformed with a recombinant DNA molecule according to any one of the preceding claims.

46. A method of inhibiting pollen development or function and thereby inducing nuclear male sterility in plants of the family Poaceae comprising the steps of:

a) developing a plant carrying a recombinant DNA molecule comprising the rye grass pollen promoter sequence or homologue or degenerate form thereof located on said molecule and a nucleotide sequence encoding a polypeptide having a deleterious function in cells derived from the family Poaceae, said nucleotide sequence transcribeable from said promoter, and said recombinant DNA molecule stably contained in pollen producing cells, and,

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b) growing said plants under conditions and for a time sufficient for their developmental stage to cause expression of said nucleotide sequence from said promoter thereby producing the polypeptide having a deleterious function on said pollen producing cells such that pollen formation is inhibited or said pollen is inactive.

47. The method according to claim 46, wherein the nucleotide sequence alternatively encodes an antisense RNA capable of inhibiting pollen formation of rendering said pollen inactive.

48. A method for desensitizing a human allergic to a grass pollen comprising administering to said human a desensitizing effective amount of Lol pI or a derivative, homologue or immunological relative thereof for a time and under conditions sufficient to effect desensitization of said human.

49. The method according to claim 48 wherein administration is by the intravenous, intramuscular, intranasal, intradermal, intraperitoneal, suppository or oral route.

50. A pharmaceutical composition useful in desensitizing a human allergic to a grass pollen comprising an effective amount of Lol pI, or a derivative, homologue or immunological relative thereof, and a pharmaceutically acceptable carrier.

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51. A peptide, polypeptide or protein comprising an amino acid sequence corresponding in whole or part to the nucleotide coding sequence represented in Figure 5 or to degenerate or homologue forms thereof.

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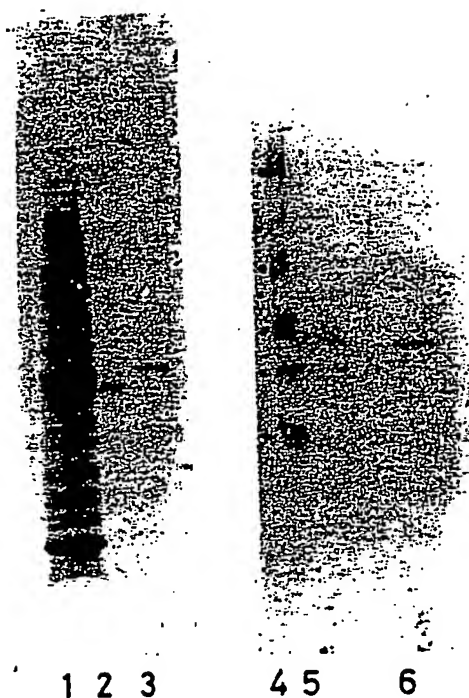


Fig.1.

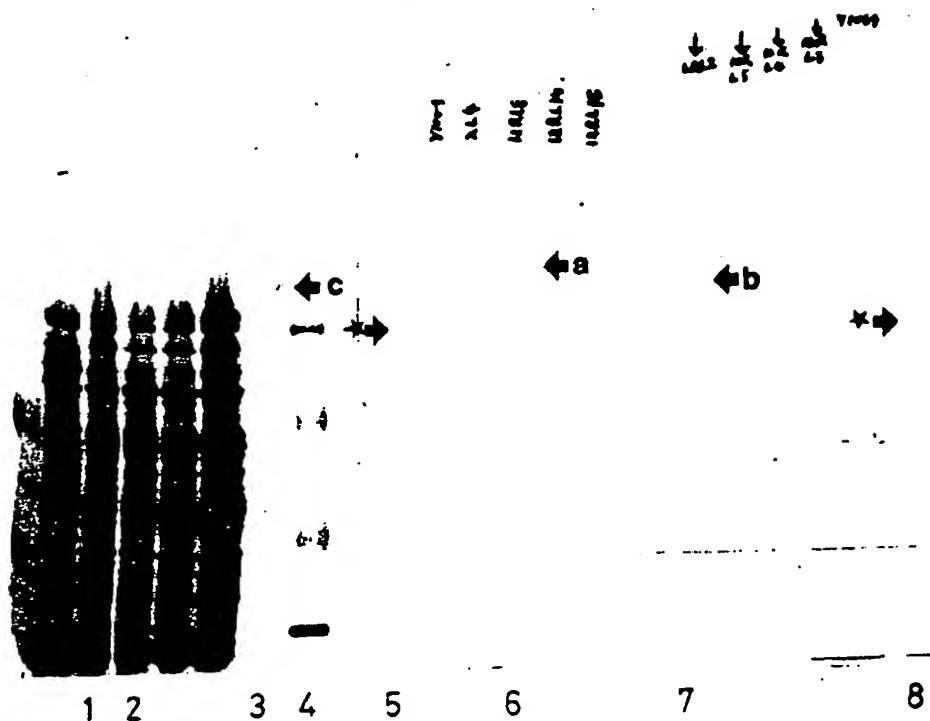


Fig.3.

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Fig. 2a.

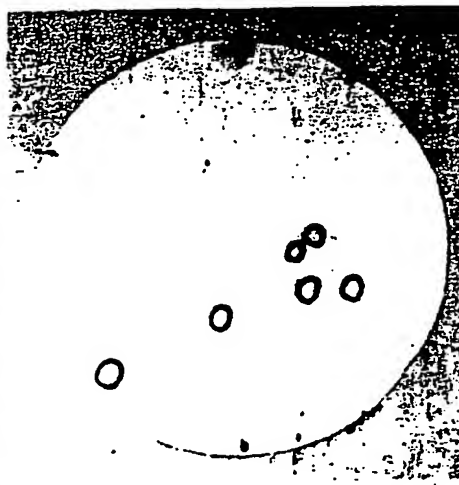


Fig. 2c.

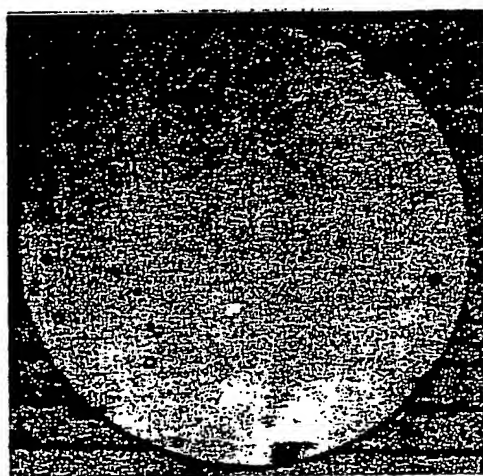


Fig. 2b.



Fig. 2d

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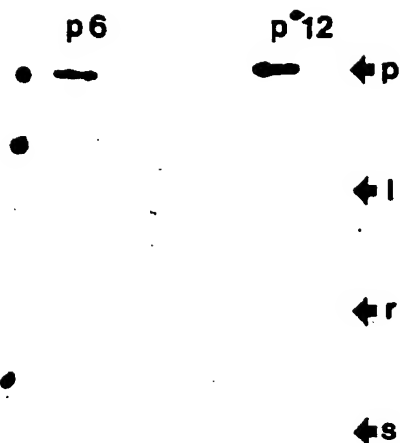


Fig.4a.



Fig.4b.

1 2 3 4

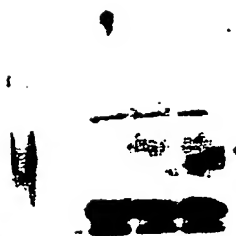


Fig.6.

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10	20	30	40	50	60
GAATTCCGCT	ATCCCTCCCT	CGTACAAACA	AACGCAAGAG	CAGCAATGGC	CGTCCAGAAC
70	80	90	100	110	120
TACACGGTGG	CTCTATTCCCT	CGCCGTGGCC	CTCGTGGGGG	CCCGGCCGCT	CCTACGCCCGC
130	140	150	160	170	180
TGACGCCCGC	TACACCCCCG	CAGCCGGGCC	ACCCCGGCTA	CTCCTGCTGC	CACCCCGGCT
190	200	210	220	230	240
GCGGCTGGAG	GGAAGGCGAC	GACCGACGAG	CAGAAGCTGC	TGGAGGACGT	CAACGCTGGC
250	260	270	280	290	300
TTCAAGGCAG	CCGTGGCCGC	CGTGCCCAACG	CCCCCTCCGC	GGACAAGTTC	AAGATCTTCG
310	320	330	340	350	360
AGGCCGCCCTT	CTCCGAGTCC	TCCAAGGGCC	TCCTCGCCAC	CTCCGCCCGCA	AGGCACCCCGG
370	380	390	400	410	420
CCTCATCCCC	AAGCTCGACA	CCGCCTACGA	CGTCGCTACA	AGGGCGAGGG	CGCCGCCACC

Fig.5(1).

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430	440	450	460	470	480
CCCGAGGCCA	AGTACGACGC	CTTCGTCACT	GCCCTCACCG	AAGCTCCGCG	TCATCGCCGG
490	500	510	520	530	540
CGCCCTCGAG	GTCCACGCCG	TCAAGCCCCG	CACCGAGGAG	GTCCCTGCTG	CTAAGATCCC
550	560	570	580	590	600
CACCGGTGAG	CTGCAGATCG	TTGACAAGAT	CGATGCTGCC	TTCAAGATCG	CAGCCACCCG
610	620	630	640	650	660
CGCCGCCAAC	GCCGCCCCCA	CCAACGATAA	GTTACCCGTC	TTGAGAGGTG	CCTTCAACAA
670	680	690	700	710	720
GGCCCTCAAT	GAGTGCACCG	GGCGGCGCTA	TGAGACCTAC	AAGTTCATCC	CCTCCCTCGA
730	740	750	760	770	780
GGCCGCGGTC	AAGCAGCCCTA	CGCCGCCACC	GTGCGCGCCG	GCCCCGAGGTC	AAGTACCCGC

Fig.5(2).

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790	800	810	820	830	840
GTCTTTGAGG	CCGCGCTCGA	CCAAGGCCAT	CACCGCCATG	ACCCAGGCAC	AGAAGGCCCG
850	860	870	880	890	900
CAAAACCCGCT	GCCGCCGCTG	CCACAGGCCG	CAACCGTTGC	CACCGCACCG	CAACCGCCGC
910	920	930	940	950	960
C--TG-C-CA	G-CCGCCGCT	GCTGGTGGCT	ACCAAAGCCT	GATCAGCTTG	CTAATATACT
970	980	990	1000	1010	1020
ACTGAACGTA	TGTATGTGCA	TGATCCGGGC	GGCGAGTGGT	TTTGTGATA	ATTAATCTTC
1030	1040	1050	1060	1070	1080
GTTTTCGTTT	CATGCAGCCG	CGATCGAGAG	GTTCATCGT	TGTAATAATT	CAATATTTT
1090	1100	1110	1120	1130	1140
TATTTCTTTT	TGAATCTGTA	AATCCCCATT	GACAAGTAGT	GGGATCAAGT	CG-CATGIAT
1150	1160	1170	1180	1190	1200
CACCGTTGAT	GCTGAGTTTA	ACGATGGGGA	GTTTATCAAA	GAATTTATTA	TTAAAAAAA
1210	1220	1230	1240	1250	1260
AAAAAAAAAA	AAAAAAAAAA	AAAAAAAAAA	AAAAAAAAAA	AAAAA	

Fig.5(3).

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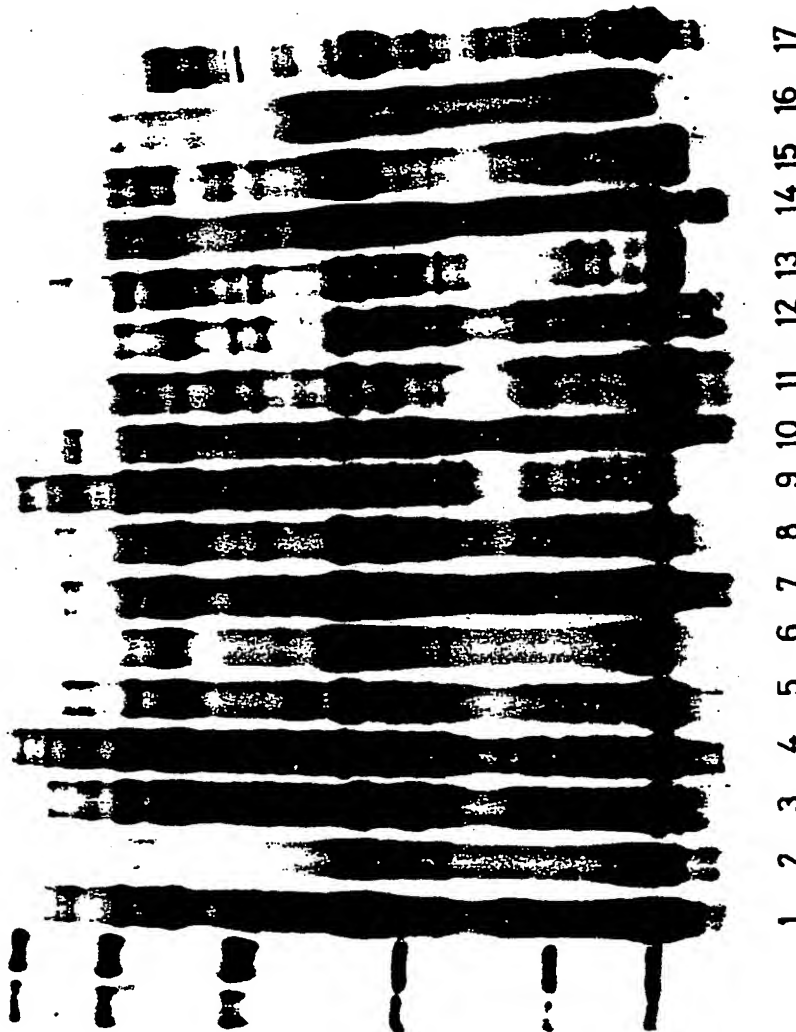


Fig. 7a.

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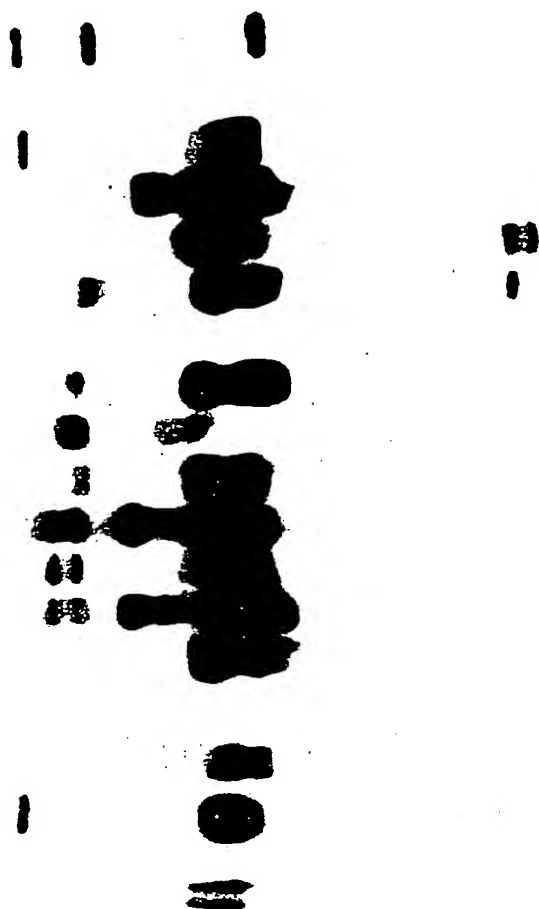
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17



Fig. 7b.

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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17



Lol p1 ➤

Fig. 7c.

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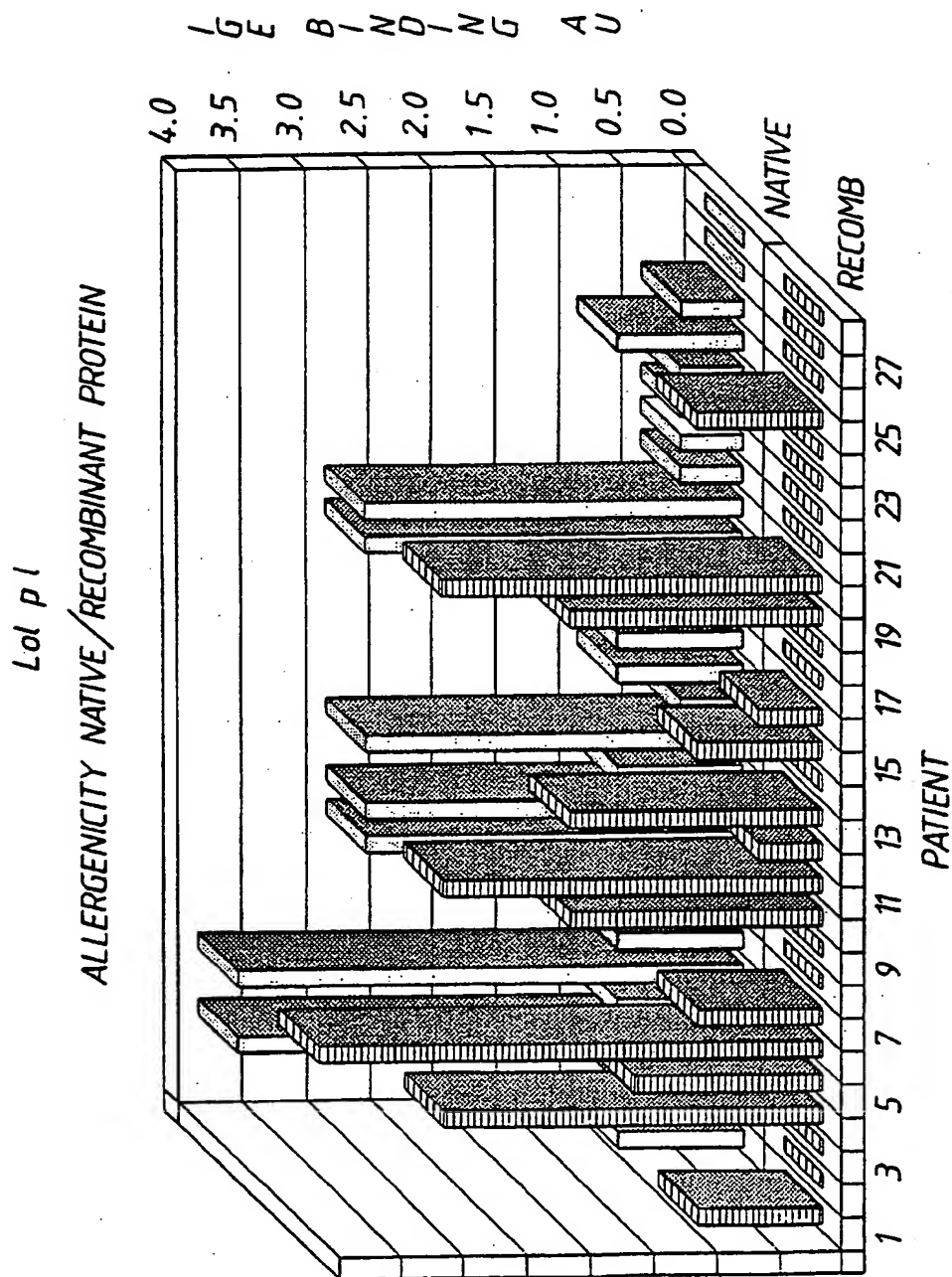


Fig. 8a.

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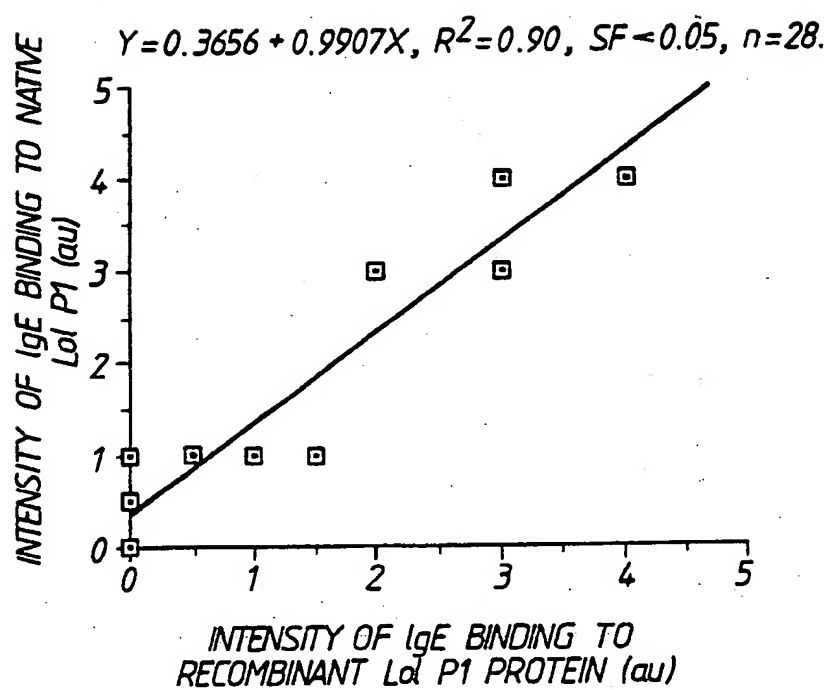
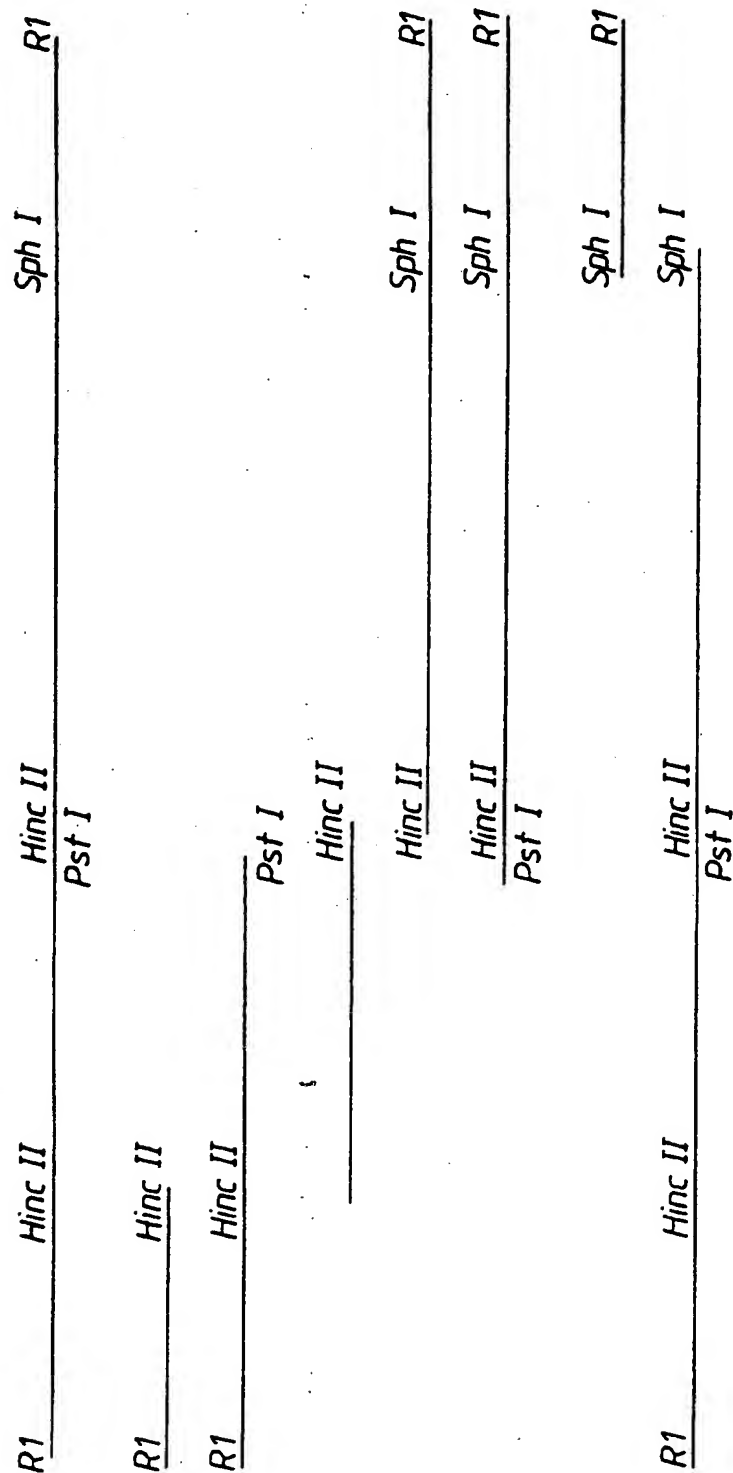


Fig.8b.

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100bp

Fig.9.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/AU 89/00123

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6		
According to International Patent Classification (IPC) or to both National Classification and IPC Int. Cl. C12N 15/00, 1/20, 5/02, C12P 21/02, 19/34, C07K 13/00, G01N 33/531, 33/532, C07K 15/12, 15/14, C12Q 1/68, A01H 1/00, C07H 21/04, A61K 39/36		
II. FIELDS SEARCHED		
Minimum Documentation Searched 7		
Classification System	Classification Symbols	
IPC	WPI, WPII, USPA, DERWENT DATA BASES: KEYWORDS RYE GRASS POLLEN ALLERGEN OR ANTIGEN; RECOMBINANT L01 PI	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8		
AU: C12N 15/00, C07K 15/12, 15/14, CHEMICAL ABSTRACTS, BIOSIS PREVIEWS, EXERPTA MEDICA, MEDLINE KEYWORDS as above		
III. DOCUMENTS CONSIDERED TO BE RELEVANT 9		
Category*	Citation of Document, with indication, where appropriate, of the relevant passages 12	Relevant to Claim No 13
X	Molecular Immunology, volume 23, no. 12, 1986 pp. 1281-1288, C.R. Kahn and D.G. Marsh, "Monoclonal Antibodies to the major <u>Lolium perenne</u> (Rye Grass) pollen allergen L01 pI (Rye I)".	(16-18, 20-23, 24-25, 28, 51)
X	Immunology, vol 59 no. 2, 1986 pp. 309-315, R. Bose <u>et al.</u> , "Production and characterization of mouse monoclonal antibodies to allergenic epitopes on L01pI (Rye I)".	(16-17, 20, 21, 23, 51)
Y		(24-28)
X	Int. Arch. Allergy, Appl. Immun. vol. 72 no. 3 pp. 243-248, 1983, I.J. Smart <u>et al.</u> , "Development of Monoclonal mouse antibodies specific for allergenic components in Ryegrass (<u>Lolium perenne</u>) pollen".	(16, 17, 19, 24-28, 51)
X	Int. Arch. Allergy, Appl. Immun. vol. 78, 1985 pp. 300-304, M.B. Singh & R.B. Knox, Grass Pollen Allergens: "Antigenic Relationships Detected Using monoclonal antibodies and Dot blotting Immunoassay".	(16-17, 19)
Y	(CONTINUED)	(24-28)
* Special categories of cited documents: 10		
"A"	document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier document but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"O"	document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed	
IV. CERTIFICATION		
Date of the Actual Completion of the International Search 30 June 1989 (30.06.89)	Date of Mailing of this International Search Report 11 JUL 1989 (11.07.89)	
International Searching Authority Australian Patent Office	Signature of Authorized Officer J H CHAN 	

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X	Journal of Allergy and Clinical Immunology vol 78 no 6 pp. 1190-1201, 1986 L.R. Friedhoff et al., "A study of the human immune response to <u>Lolium perenne</u> (Rye) pollen and its components, L01 pI and L01 pII (Rye I and Rye II)".	(16, 20-21, 23, 24-28, 51)
X,P	Tissue Antigens vol 31 no 4 pp 211-219 (1988) L.R. Friedhoff et al., "Association of HLA-DR3 with human immune response to L01 pI and L01 pII allergens in allergic subjects".	(20-21, 23-28, 50, 51)
X	Int. Arch. Allergy, Appl. Immun. volume 85 no.1 pp 104-108 (1988) R.B.cook et al "Induction of Allergen -Specific T-Cells by conjugates of N-formyl-methionyl-leucyl-phenylalanine and Rye grass pollen extract".	(48-51)
Y,P	Chemical abstracts vol. 108 issue no 23 1988. Wheeler A.W. et al "Retained T-cell reactivity of rye grass pollen extract following cleavage with cyanogen bromide and nitrothiocyanobenzoic acid". (CONTINUED)	(48-51)

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE 1

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

- 1.[X] Claim numbers ...48-50, because they relate to subject matter not required to be searched by this Authority, namely:
Rule 39(iv) Methods for treatment of the human or animal body by surgery or therapy.
- 2.[] Claim numbers ..., because they relate to parts of the international application that do comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
- 3.[] Claim numbers ..., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4 (a):

VI. [X] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING 2

This International Searching Authority found multiple inventions in this international application as follows:

Group I - Claims 1-14, 20-28 and 42 are directed to recombinant known L01 pI protein, cDNA thereof, expression of cDNA in transformed host, cDNA as probe, use of recombinant protein.

(CONTINUED)

1. [] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. [] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. [X] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
1-14, 20-28, 42, 15-19, 48-50 and 51.
4. [] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- [] The additional search fees were accompanied by applicant's protest.
[] No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Journal of Chromatography, vol 370 issue no. 1 pp. 165-172 (1986) BRIEVA A. and RUBIO N. "Rapid purification of The Main allergen of <u>Lolium perenne</u> by high performance liquid chromatography".	(51)

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